PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTI-ARTHRITIC ACTIVITY ON THE BARK OF *Sterculia foetida* Linn., AND ISOLATION OF BIOLOGICAL MARKER

A Dissertation submitted to

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In partial fulfilment of the requirements for the award of the degree of

MASTER OF PHARMACY IN

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Submitted by

Reg. No. 261420660

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APRIL – 2016

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CERTIFICATE

This is to certify that this dissertation work entitled **''PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTI-ARTHRITIC ACTIVITY ON THE BARK OF *Sterculia foetida* Linn., AND ISOLATION OF BIOLOGICAL MARKER''** Submitted by Reg. No. 261420660 in partial fulfilment of the requirements for the award of the degree in MASTER OF PHARMACY IN PHARMACOGNOSY by the **Tamilnadu Dr. M.G.R. Medical University**, Chennai, is a bonafide record of the work done by her in the department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai. During the academic year 2015-2016 under the guidance of **DR. R.VADIVU M.Pharm., Ph.D.,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai-600003.

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Dedicated to my family,

guide and friends

Introduction

1. INTRODUCTION

Arthritis is a form of joint disorder that involves inflammation of one or more joints, synovial tissue inflammation, associated with warm swollen and painful joints, it also affects the underlying bone and cartilage. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility. The structures especially affected are joints, tendons, ligaments, bones and muscles. Arthritis affected 15% of people that is over 180 million people, in India.[1] It is not a hereditary disease. It can develop at any age but most commonly occurs between the age of 40 and 60. The most commonly affected joints are the small joints in the fingers, thumbs, wrists, feet and ankles. Pain medications, steroids and NSAID's are frequently used to help with symptoms. Arthritis is one of those conditions on which billions of dollars are spent every year for the treatment and research.

CAUSES FOR RHEUMATOID ARTHRITIS[2]

The causes for rheumatoid arthritis are not properly known. It's an auto immune problem which means the body defence system start attacks its own joints and organs. Immune system mistakenly sends antibodies to the lining of your joints, it attack the tissues sourrounding the joints. This causes the thin layer of cells covering the joints to become sore and inflamed. Inflammation in the joints releases chemicals that thicken the synovium, it leads to gradually the loss of shape of joint and alignment and destroy the joints completely. Certain genes have been identified that increase the risk of rheumatoid arthritis. Hormonal changes and smoking increases the risk of developing rheumatoid arthritis.

CLINICAL MANIFESTATION

The most common systemic manifestation of rheumatoid arthritis is anaemia, which occurs more frequently in the early stage of the disease. Additionally haemoglobin levels are inversely correlated with IL-6 levels. IL-6 levels are required for the induction of hepcidine during inflammation and rapidly induces hypoferraemia in humans. Release of TNF-α, IL-6 and IL-1 from synovial tissue, including adipose tissue, skeletal muscle, liver and the vascular endothelium. Rheumatoid arthritis related dyslipidaemia is characterized by low total and high-density lipoprotein

(HDL), cholesterol, elevated triglyceride and lipoprotein levels. It also associated with risk of future myocardial infarction.

PATHOGENESIS AND PATHOPHYSIOLOGY[3]

The earliest event in rheumatoid arthritis pathogenesis is activation of the innate immune response, which includes the activation of dendritic cells by exogenous material and autologous antigens. Antigen presenting cells, including dendritic cells, macrophages and activated B cells, present arthritis-associated antigens to T cells.

Concurrently, $CD4^+$ T cells that secrete IL-2 and IFN-Y infiltrate the synovial membrane. As noted previously, most patients with rheumatoid arthritis carry the epitope of the HLA-DR β-chain that confers binding of specific peptides and affects antigen presentation to TCRs. Disease associated HLA-DR alleles may present arthritis-related peptides, leading to stimulation and expansion of auto antigen specific T cells in the joints and lymph nodes. B cells contribute to rheumatoid arthritis pathogenesis not only through antigen presentation, but also through the production of antibodies, auto-antibodies and cytokines. RF and anti-CCP autoantibodies are common in patients with rheumatoid arthritis. B lymphocytes express cell surface proteins, including immunoglobulin and differentiation antigens such as CD20 and CD22. Autoantibodies can form larger immune complexes that can further stimulate the production of pro-inflammatory cytokines, including TNF-α, through complement and Fc-receptor activation.

T and B- cells activation result in increased production of cytokines and chemokine's, leading to a feedback loop for additional T-cell, macrophage and B-cell interactions. In addition to antigen presentation, macrophages are involved in osteoclast genesis and are a major source of cytokines, including TNF-α, IL-1 and IL-6. Within the synovial membrane there is a great increase in activated fibroblast-like synoviocytes, which also produce inflammatory cytokines, PGs and MMPs. Synoviocytes contribute to the destruction of cartilage and bone by secreting MMPs into the SF and by direct invasion into these tissues.

FIG NO: 1 PATHOGENESIS OF RHEUMATOID ARTHRITIS

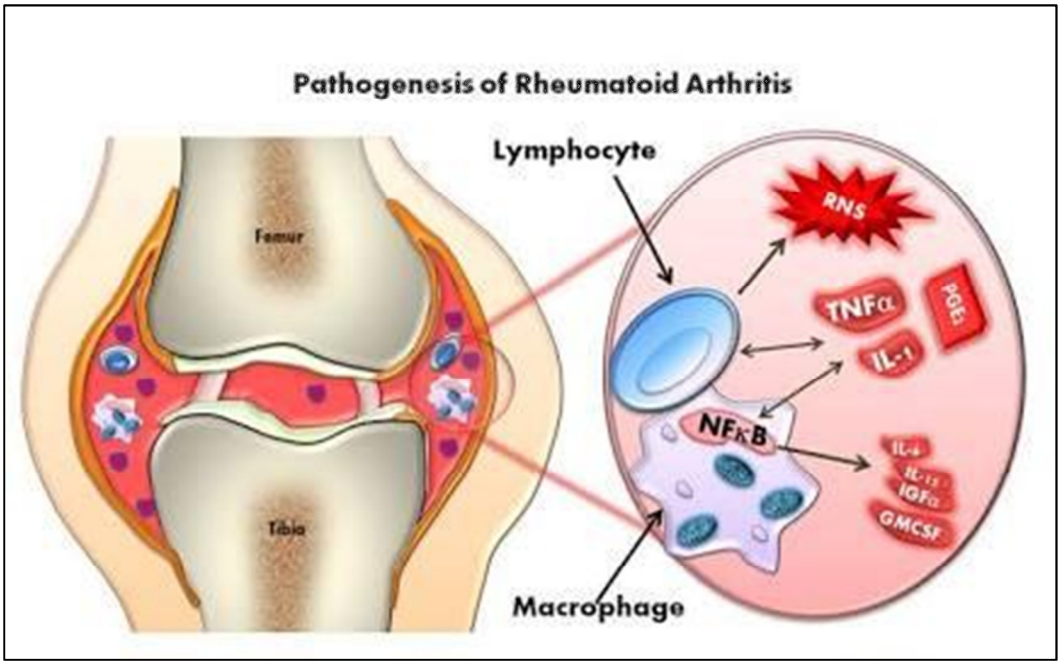
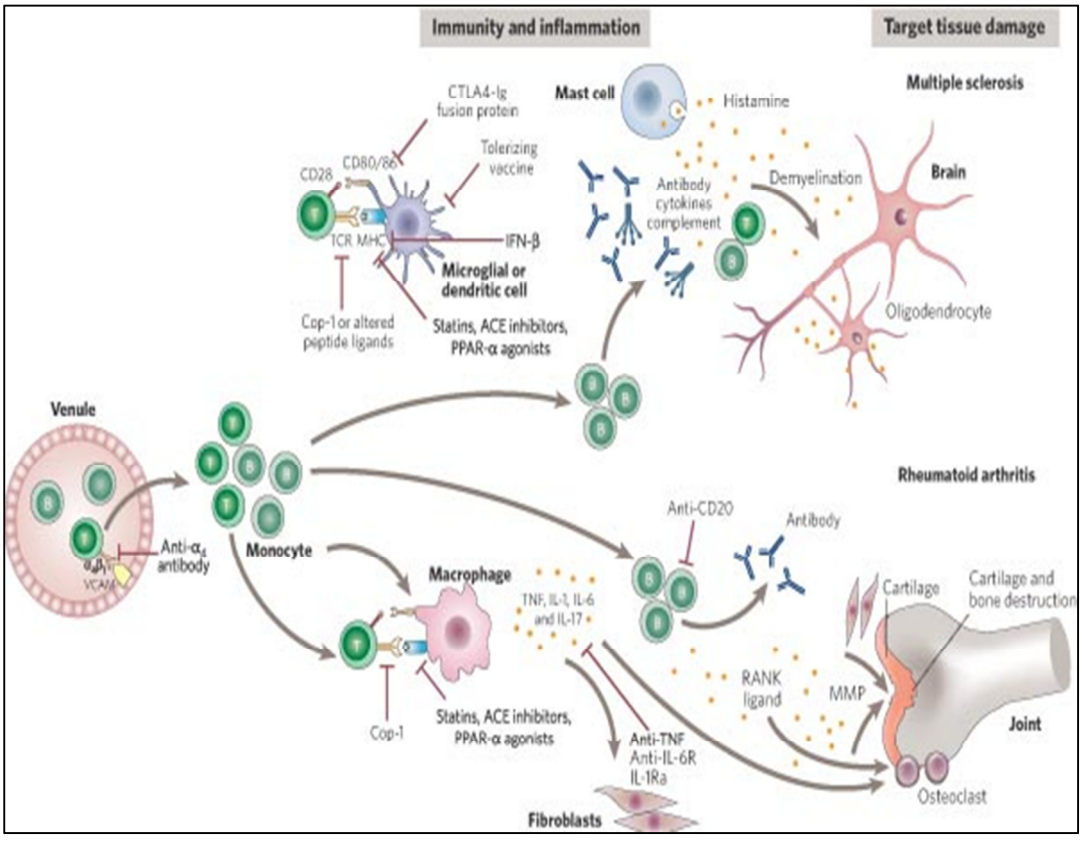


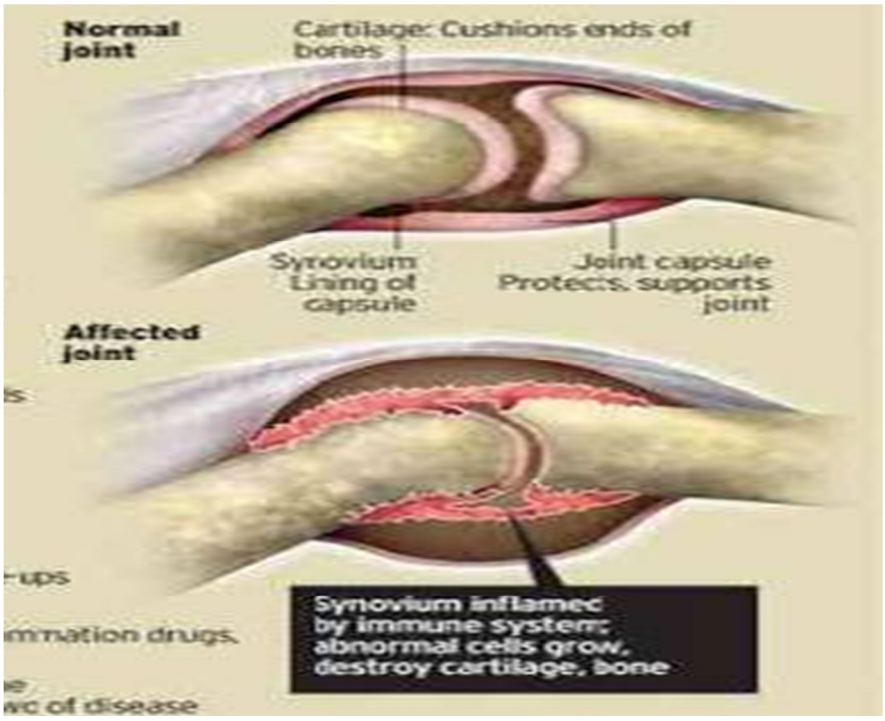
FIG NO: 2 SCHEMATIC REPRESENTATION OF RHEUMATOID ARTHRITIS



SIGNS AND SYMPTOMS[4]

- Pain, swelling and stiffness of affected joints. The stiffness is usually worse in the morning.
- Inflammation in the joints and sometimes in various organs of the body, such as the lungs, eyes etc,.
- Morning stiffness that may last for hours
- Pain spreads to the wrists, knees, ankles, elbows, hips and shoulders.
- Erosions or periarticular osteopenia in hand or wrist joints.
- Reduced ability to move the joint.
- Redness of the skin around the joint during mornings.

FIG NO: 3 NORMAL AND ARTHRITIC JOINT



MANAGEMENT OF ARTHRITIS[5]

The focus of treatment for arthritis is to control pain, minimize joint damage and improve or maintain function and quality of life. According to the severity of disease the management of arthritis involves as follows,

- ❖ Medications
- ❖ Non-pharmacologic therapies
- ❖ Physical or occupational therapy
- ❖ Splints or joint assistive aids
- ❖ Weight loss
- ❖ Surgery

SYNTHETIC DRUGS FOR THE TREATMENT OF ARTHRITIS[6]

The following types of drugs can be used to treat rheumatic diseases and related conditions.

- **Analgesics**—pain relievers such as acetaminophen.
- **Topical analgesics**—creams or ointments that are rubbed into the skin over sore muscles or joints to relieve pain.
- **Non-steroidal anti-inflammatory drugs (NSAIDs)**—pain relievers such as Ibuprofen, Naproxen sodium and ketoprofen are available over the counter. Some NSAIDS are available only with a prescription.
- **Disease-modifying anti-rheumatic drugs (DMARDs)**—a family of medicines used to treat conditions like rheumatoid arthritis and spondylitis. These drugs work by slowing or stopping the immune system from attacking the joints.
- **Hyaluronic acid substitutes**—these medicines mimic a naturally occurring body substance that moistens joints to help them move better. Usually a person receives three to five injections directly into the affected knee or hip to help relieve pain and provide flexible joint movement.
- **Nutritional supplements**—these products include S-adenosylmethionine (SAM-e) for osteoarthritis and fibromyalgia, dehydroepiandrosterone (DHEA) for lupus and glucosamine and chondroitin sulfate for osteoarthritis. Patients should read reports on how safe and effective a supplement is before taking it. Many have not been proven helpful in scientific studies. The U.S. Food and Drug Administration does not regulate supplements the way it monitors drugs.

HERBAL TREATMENT FOR ARTHRITIS[7, 8]

Man was completely depending on the medicinal plants for the treatment of disease before the discovery of synthetic drugs. Alternative systems of medicine are still curing arthritis instead of treating symptoms. In an effort to gain relief and take a natural approach, more arthritis patients are seeking herbal remedies than ever before. Certain herbs may have anti-inflammatory properties that can help with rheumatoid arthritis, as well as the ability to reduce pain in all forms of the disease.

SOME OF THE HERBS USED IN THE TREATMENT OF ARTHRITIS

TABLE NO: 1 NATURAL HERBS FOR TREATING ARTHRITIS

S.NO	NATURAL HERBS FOR TREATING ARTHRITIS
1.	*Aloe vera*
2.	Boswellia
3.	Cat's claw
4.	Eucalyptus
5.	Ginger
6.	Green tea
7.	Thunder god vine
8.	Turmeric
9.	Willow bark
10.	Clove
11.	Devils claw
12.	Psammosile
13.	Almonds
14.	*Swertia chiraytia*
15.	*Nyctanthes arbor*

Considering the importance of herbal medicine in the treatment of arthritis, the present study was aimed to identify a plant available abundantly in India which has the potential anti-arthritic agent for the treatment of arthritis.

Review of Literature

REVIEW OF LITERATURE

A thorough literature survey was carried out in-order to identify the plant which is traditionally used in the treatment of arthritis. Among so many herbs available to treat arthritis. *Sterculia foetida* was selected for the present study because it is traditionally used to treat arthritis but not yet proven scientifically[9, 10].

Literature review of *Sterculia foetida* Linn., was carried out to find out the research work on this plant. The review of literature showed that many studies were done on different part of this plant. This review is made to confirm that anti-arthritic activity on the bark was not yet proven scientifically. Hence further studies were easy to carry out.

PHARMACOGNOSTICAL REVIEW

- Sonia Mitra *et al*., (2014) studied the taxonomic significance of petiole anatomy of Sterculiaceous species distributed in northeast India.[11]
- Anitha *et al*., (2002) carried out the study on shoot regeneration from hypocotyl tip explants of *Sterculia foetida* Linn., derived from the seedlings.[12]

PHYTOCHEMICAL REVIEW

- Christie WW *et al.,* (2014) studied the Fatty acids: Natural alicyclic, structures, occurrence and biochemistry.[13]
- Kale SS *et al*., (2011) carried out the Analysis of fixed oil from *Sterculia foetida* Linn.,[14]
- Niran Vipunngeum *et al*., (2009) studied the Fatty acids of S*terculia foetida* seed oil.[15]
- Xia P *et al*., (2009) studied the Chemical constituents from the leaves of *Sterculia foetida* Linn.,[16]

PHARMACOLOGICAL REVIEW

- Ravichandran M *et al*., (2015) studied the *In-vitro* antioxidant, anticancer and phytochemical screening of Sengathari Thylum-A polyherbal siddha formulation.[17]

- Shazia Hussain S *et al*., (2014) conducted the preclinical of anti-diabetic and Anti-hyperlipidemic activity of methanolic extract of *Sterculia foetida* Linn., leaves by using Wistar albino rats.[18]
- Raja TAR *et al*, (2014) carried out the evaluation of anticonvulsant effect of *Sterculia foetida* (Pinari) in Pentylenetetrazole (PTZ) and maximal Electrical shock induced convulsions in albino rats.[19]
- Shivakumar Singh P *et al.,* (2014) studied the anti-dermatophytic activity of low polar petroleum ether and inter polar methanolic extract of *Sterculia foetida* Linn.,[20]
- Narsing Rao Galla *et al*., (2012) studied the *In-vitro* antioxidants activity of *Sterculia foetida* Linn., seed methanol extract.[21]
- Pierangeli G *et al.,* (2011) performed the antimicrobial activity, cytotoxicity and phytochemical screening of *Ficus saptic* Burn and *Sterculia foetida* Linn.,[22]
- Majumdar AM, *et al*., (2011) conducted the Pharmacological studies on *Sterculia foetida* leaves.[23]
- James Perfield *et al*., (2011) performed the study Plant oil may hold key to reducing obesity related medical issues.[24]
- Usharani P *et al*, (2009) studied the Toxic and antifeedant activity of *Sterculia foetida* Linn., seed crude extract against *Spodoptera litura* F. and *Achaea janata* L.,[25]
- Edward T *et al*, (1964) carried out the study on Delay of sexual maturity of the female rat.[26]

- Noamesi BK *et al*, (1986) carried out Preliminary report on the bronchodilator properties of the aqueous stem bark extract of *Sterculia foetida.*[27]
- Scarpelli DG *et al*., (1974) studied the Mitogenic activity of sterculic acid, a cyclopropenoid fatty acid.[28]

PHARMACEUTICAL REVIEW

- Mahakalkar NG *et al*., (2013) carried out study of Zolmitriptan nasal In-situ gel using *Sterculia foetida* Linn., gum as natural muco-adhesive polymer.[29]
- Bindhu CH *et al*., (2011) Prepared and evaluation of bio-diesel from *Sterculia foetida* seed oil.[30]
- Amit Ashok *et al*., (2008) carried out the Evaluation of *Sterculia foetida* gum as controlled release excipients.[31]

Plant Profile

3. PLANT PROFILE[32, 33]

Plant name	:	*Sterculia foetida* Linn.,
Common name	:	Wild Almond, Java Olive.
Family	:	Sterculiaceae

VERNACULAR NAMES

- **Tamil - Kutiraippitukkan,**
- Bengali - Jungli Badam
- Hindhi - Jangli Badam
- Kannada - Bhatala Penari,
- Konkani - Kuvem ruk,
- Malayalam - Pinar,
- Marathi - Punava,
- Telugu - Adavibadamu,
- Sanskrit - Putidaru.

TAXONOMICAL STATUS

- Kingdom : Plantae
- Sub Kingdom : Tracheophyta
- Division : Mangoliophyta
- Class : Magnoliopsida
- Sub class : Dilleniidae
- Order : Malvales
- Family : Sterculiaceae
- Genus : *Sterculia*
- Species : *foetida*

HABITAT : East Asia-India, Sri-Lanka, Myanmar and Thailand.

FIG NO: 4 ENTIRE PLANT OF *Sterculia foetida* Linn.,

PLANT DESCRIPTIONS[34, 35]

TABLE NO: 2 PLANT DESCRIPTIONS

S.NO	PARTS	IMAGES	DESCRIPTION	CHEMICAL CONSTITUENTS	USES
1.	**Leaves**		7-9 leaflets(crowded) of 10-17cm long, petiole 12.5-23cm long	Procyanidin, scutellarein and luteolin and also consists, of taraxerol, n-otacosanol and beta-sitosterol	Aperient, diuretic, abortifacient, skin disease, anti-diabetic activity, anti-convulsant, antioxidant, anti-cancer activity, CNS depressant and anti-inflammatory activity.
2.	**Seed**		ovoid-oblong, black	Cyclopropene fatty acids-Sterculic acid and Malvalic acids	Seed oil-skin disease and rheumatism.
3.	**Bark**		Smooth and grey, Thickness 2.5-3cm, fibrous in nature	Secutellarin, betulin, lupeol and betullic acid.	Rheumatism, broncho dilator, diaphoretic and diuretic.

S.NO	PARTS	IMAGES	DESCRIPTION	CHEMICAL CONSTITUENTS	USES
4.	**Root**		Thick, brown in colour	Leucoanthocyanidin-3-alpha-L-rhamnopyranoside and quercetin and rhamnoside.	Anti-inflammatory.
5.	**Flower**		Reddish orange in colour	Contains fatty acids	-

Rationale for selection

Of the plant

4. RATIONALE FOR SELECTION OF THE PLANT

Arthritis is a chronic disease which affects 15% of the people in India. The research work is decided to study the herbal plant drug which cures arthritis and reduces side effects.

Traditionally, *Sterculia foetida* is used in the treatment of various disorders by the traditional healers it is also used as aperient, diuretic, abortifacient, skin disease, anti-diabetic activity, anti-convulsant, antioxidant, CNS depressant and anti-inflammatory activity.

The bark has a claim to treat arthritis which is not proven scientifically. Hence the present work was under taken to establish the effects of *Sterculia foetida* bark in the treatment of arthritis.

Aim and Objective

5. AIM AND OBJECTIVE

AIM

To evaluate the Pharmacognostical, Phytochemical and Anti-arthritic activity on the bark of *Sterculia foetida* Linn.,

OBJECTIVE

Pharmacognostical studies: Establishment of Pharmacognostical standards for the bark of *Sterculia foetida* Linn.,

Phytochemical studies: To determine the phytochemical constituents and to isolate and identify the biomarkers present in the plant

Pharmacological studies

- To select the active extract by *in-vitro* anti-arthritic and anti-oxidant studies
- To evaluate *in-vivo* anti-arthritic activity on the bark of *Sterculia foetida* Linn.,

Plan of Work

PLAN OF WORK

1. **COLLECTION OF PLANT MATERIAL**
2. **AUTHENTICATION**
3. **PHARMACOGNOSTICAL STUDIES**
    - **Macroscopy**
    - **Microscopy**
        - **Histochemical studies**
        - **Powder microscopy**
        - **Quantitative microscopy – Linear measurement**
    - **Physiochemical constants**
4. **PHYTOCHEMICAL STUDIES**
    - **Preparation of extracts**
    - **Preliminary phytochemical analysis**
    - **Quantitative estimation of phytoconstituents**
    - **Fluorescence analysis**
    - **Thin layer chromatography**
    - **High performance thin layer chromatography**
5. **SELECTION OF ACTIVE EXTRACT**
    - ***In-vitro* anti-oxidant activity**
        - **DPPH Assay**
        - **Nitric oxide scavenging assay**
    - ***In-vitro* anti-arthritic activity**
        - **Protein Denaturation Method**
6. **ACUTE TOXICITY STUDIES**
7. ***IN-VIVO* ANTI-ARTHRITIC ACTIVITY**
8. **ISOLATION AND IDENTIFICATION OF BIOLOGICAL MARKER**

Pharmacognostical studies

6. PHARMACOGNOSTICAL STUDIES

6.1. MATERIAL AND METHODS

COLLECTION OF PLANT MATERIAL

The fresh healthy bark of *Sterculia foetida* Linn., was collected in the month of June at Madras Medical College, Chennai 03

AUTHENTICATION OF THE PLANT BARK

The plant material was authenticated by **Dr. D. Aravind. M.D.(S), Msc. Assistant Professor,** Medicinal Plants, National Institute of Siddha (Govt. of India), Tambaram. A voucher specimen (voucher specimen no: 10) is deposited in the Department of Pharmacognosy, Madras Medical College, Chennai.

MACROSCOPIC EVALUATION

Various organoleptic characters like colour, odour, taste and nature of the bark like its size, shape, surface, fracture and thickness were observed for the determination of safety, efficacy and purity of crude drugs.

MICROSCOPIC EVALUATION[36-39]

Fixation of bark

The bark was cut and fixed in FAA solution (Formalin 5ml + Acetic acid 5ml + 90ml of 70% Ethanol). The specimen was dehydrated after 24 hours of fixing. The bark was graded with series of tertiary butyl alcohol, as per the standard procedure.

It was carried out by gradual addition of 58 – 60º C of melting pointed paraffin wax until Tertiary butyl alcohol (TBA) solution attained super saturation. The specimens were cast into paraffin blocks.

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 - 12µ. De-waxing of the sections was done by customary procedures. The sections were stained with haematoxilin. The stained sections were viewed under microscope.

Photomicrographs

Microscopic descriptions of tissues were supplemented with photo micrographs whenever necessary. Photo micrographs of section were taken at different magnification for focusing of different microscopical parts of the bark.

POWDER MICROSCOPY[40, 41]

The shade dried bark was powdered and used for powder microscopic analysis. The organoleptic characters were observed and to identify the different microscopical characteristic features various staining reagent were used. Powder was stained with 1% phloroglucinol in 90% ethanol, concentrated hydrochloric acid and observed under microscope. Powder analysis is used for the detection of characteristic structures and various cell components.

HISTOCHEMICAL COLOR REACTIONS[42]

The transverse sections of bark were stained with different staining reagent such as Phluoroglucinol, Iodine, Ferric chloride, Dragendroff's reagent, toluidine blue and Picric acid to observe and localize the presence of lignin contents, starch, tannins alkaloids, flavonoids and proteins as per the standard procedure. The stained sections were then washed in water to remove the excess stain and observed under a microscope.

QUANTITATIVE MICROSCOPY[43]

LINEAR MEASUREMENTS OF FIBRES

The length and width of the fibers present in the bark were observed under microscope. This quantitative analysis will be helpful in the identification of the drug.

The first step involved in this is calibration of the eyepiece micrometer using the stage micrometer. For determining the calibration factor, the eyepiece is removed from the microscope, then the lens is unscrewed and in the ridge the eyepieces micrometer is placed. The lens is then replaced. The stage micrometer is then placed on the stage of the microscope and focused under high power with the eyepiece coincides with each division of stage micrometer and calculate the calibration factor using the standard formula.

The stage micrometer is replaced with the slide containing the powdered drug. The slide is prepared by using the bark powder on a slide is treated with a drop of phloroglucinol and conc. Hydrochloric acid and viewed under microscope. The width and length of fibers is measured by focusing them on the lines of the eyepiece micrometer. Note the no. of divisions covered by the length and width of the fibers.

PHYSIOCHEMICAL ANALYSIS[43-45]

The shade dried powdered bark of *Sterculia foetida* Linn., was used for the analysis of various physiochemical parameters which is useful in the determination of quality and purity of crude drugs. Total ash, extractive values, loss on drying, foaming index, swelling index and foreign organic matters were determined as per the standard WHO guidelines which is very much useful in the determination of quality and purity of the crude drugs.

DETERMINATION OF ASH VALUES

The residue remaining after incineration is the ash content of the drug, which simply represents the inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration.

TOTAL ASH

Silica crucible was heated to red hot for 30 minutes and it was allowed to cool in desiccators. About 2gm of powdered sample was weighed accurately and evenly distributed in the crucible. Dried at 100 – 105ºC for 1 hour and ignited to constant weight in a muffle furnace at 600±25ºC. The crucible was allowed to cool in a desiccator. The percentage yield of ash with reference to the air dried substance was then calculated by the formula

Water soluble ash

The total ash was boiled for 5min with 25ml of water. The insoluble matter was then collected in an ash less filter paper. It was washed with hot water and ignited for 15min at a temperature not exceeding 450ºC. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represented the

water soluble ash, the percentage of water soluble ash with reference to the air dried substances was calculated with reference to the air dried material.

Acid insoluble ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth

Procedure

To the total ash obtained previously, 25ml of dilute hydrochloric acid was added, covered with a watch glass and boiled gently for 5min on a burner. The watch glass was rinsed with 5ml of hot water and these washings were added to the crucible. The insoluble matter was collected on an ash less filter paper by filtration and the filter paper was rinsed repeatedly with hot water until the filtrate is neutral and free from acid. Filter paper containing the insoluble matter was transferred to the crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450-500ºC. The silica crucible was removed from the muffle furnace and allowed to cool in a desiccator for 30min, and then weighed without delay. The content of acid insoluble ash was calculated

Sulphated ash

About 3gm of air-dried substance was ignited gently at first in a crucible, until the substance was thoroughly charred. Then the residue was cooled, moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800 ± 25ºC, until all the black particles were disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed. The percentage of sulphated ash with reference to the air- dried substance was then calculated.

DETERMINATION OF EXTRACTIVE VALUES

This method is used to determine the amount of active constituents in a given amount of plant material when extracted with solvents. Extractive values are useful for the evaluation of phytoconstituents especially when the constituents of a drug

cannot be readily estimated by any other means. Further these values indicate the nature of the active constituents present in a crude drug.

Determination of water soluble extractive

About 5gm of the powder was weighed and macerated with 100ml of chloroform water (95ml distilled water and 5ml chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. 2 ml of alcohol was added to the residue and it was dried at 105ºCfor 1 hour in the hot air oven and cooled in desiccators for 30min and weighed. The process was repeated till a constant weight was obtained; the percentage of water soluble extractive value with reference to the air dried drug was calculated.

Determination of alcohol soluble extractive

The alcohol soluble extractive value is also indicative for the same purpose as water soluble extractive value. The solvent strength of alcohol varies from 20-90%v/v. The solvent strength has to be chosen depending upon the strength of alcohol used for the extraction of powdered drug.

Procedure

About 5gm of the powder was weighed and macerated with 100ml 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. It was dried at 105ºC for 1hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air dried drug was calculated.

Determination of non-volatile ether soluble extractive (fixed oil content)

Accurately weighed quantity of the drug was transferred to an extraction thimble and extracted with solvent ether or petroleum ether (Boiling Point 40 - 60ºC) in a Soxhlet for 6 hours. The extract was filtered into a tarred evaporating dish, evaporated and dried at 105°C to constant weight. The percentage of non-volatile ether soluble extractive value with reference to the air dried drug was calculated.

Determination of volatile ether soluble extractive

About 2gm of powdered drug was accurately weighed and extracted with anhydrous diethyl ether in a continuous extractive apparatus for 20hours. The ether solution was transferred to tarred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18hours and the total ether extract was weighed. The extract was heated gradually and dried at 105ºCto constant weight. The loss in weight represents the volatile portion of the extract.

LOSS ON DRYING

Accurately weighed quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents was weighed accurately. The loaded crucible and the lid were placed in the drying chamber (105ºC). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a desiccator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

DETERMINATION OF FOAMING INDEX

About 1gm of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at temperature 80-90ºC for about 30min. It was then cooled and filtered into a volumetric flask and sufficient water was added through the filtrate to make up the volume to 100ml. The decoction was poured into 10 stopper test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, 4ml up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were

stoppered and shaked in a length wise motion for 15 seconds, two shakes per second. Allowed to stand for 15min and the height of the foam were measured. The results are assessed as follows: if the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result. The height of the foam is more than 1cm in every tube the foaming index is over 1000. In this case repeat the determination using a new series of dilution of the decoction in order to obtain a result. Calculate the foaming index using the following formula.

Foaming index = 1000/a

Where, **a** is the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

DETERMINATION OF SWELLING INDEX

The swelling index is the volume in ml occupied by the swelling of 1gm of plant material under specified conditions.

Procedure

A specified quantity of the plant material was accurately weighed and transferred into a 25ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion about 125mm, marked in 0.2ml divisions from 0 to 25ml in an upward direction. Unless otherwise indicated in the test procedure, add 25ml of water and shake the mixture thoroughly every 10min for 1hour, allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material was measured including any sticky mucilage. Calculate the mean value of the individual determination, related to 1gm of plant material.

FOREIGN ORGANIC MATTER

The weighed quantity of bark powder was examined for the determination of foreign organic matter by inspection with the use of lens and calculated.

6.2. RESULTS AND DISCUSSION

The results of pharmacognostical studies are as follows.

MACROSCOPICAL STUDIES

Organoleptic evaluation

Colour - Grey

Taste - Bitter

Odour - Odourless

Texture - Smooth

Morphological evaluation

Appearance - Outer bark is smooth grey in colour

Inner bark is fibrous brown in colour

Shape - Single quill

Length - 13-15cm

Thickness - 2.5-3cm

Fracture - Fibrous

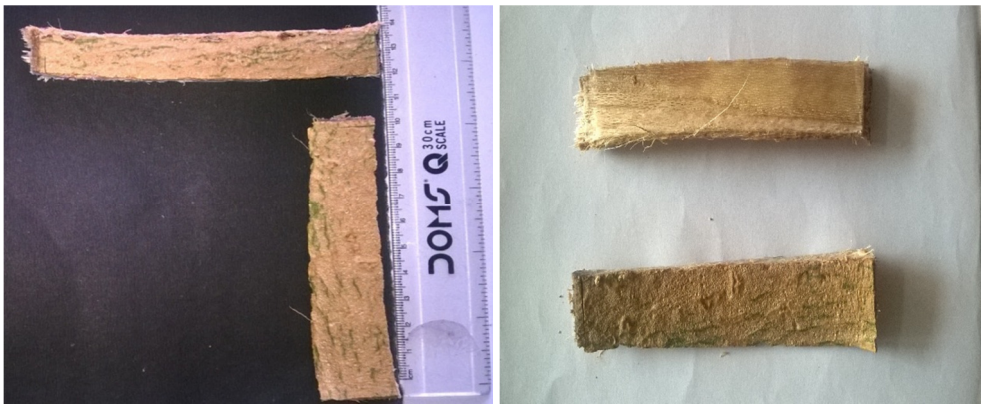


FIG NO: 5 & 6 MORPHOLOGY OF THE BARK OF *Sterculia foetida* Linn.,

MICROSCOPY OF BARK

The transverse section of bark of *Sterculia foetida* Linn., showed different types of cells. Cork cells, cortex, cork cambium, sclerenchyma, endodermis, phloem cells, calcium oxalate crystals, medullary rays, pericyclic fibres were observed. These characters are observed in different magnification lenses. The following characters were seen and reported by photographically.

FIG NO: 7 ENTIRE TRANSVERSE SECTION OF BARK (4X)

Cork

It consists of 10-15 layers of rectangular, regularly arranged cells in radial rows and have some reddish brown contents.

Cortex

It consists of parenchyma cells in which scattered sclereids either isolated or in groups.

Medullary rays

It consists of parenchymatous cells run diagonally and extent from pith(medulla) to cortex through secondary xylem and secondary phloem. Width of the rays are 4-5 cells(multiseriate) wavy.

Phloem fibers

It is small, lignified and polygonal in shape.

Sclerenchymatous cells

The sclereids are which are lignified and pitted. Inner and radial walls of the sclereids are more thicker than the outerwalls and appears as U shape.

Calcium oxalate crystals

Prism like calcium oxalate crystals are observed in the cortical sclerenchyma.

ENLARGED MICROSCOPY OF BARK

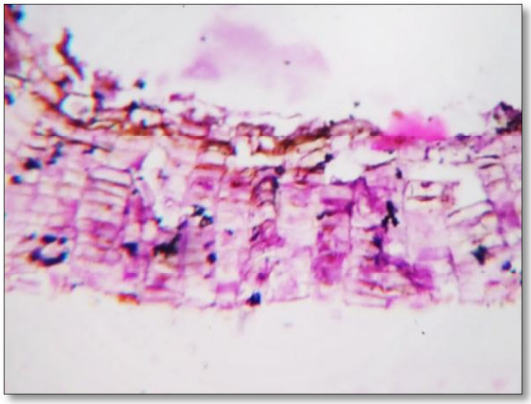


FIG NO: 8 CORK ENLARGED

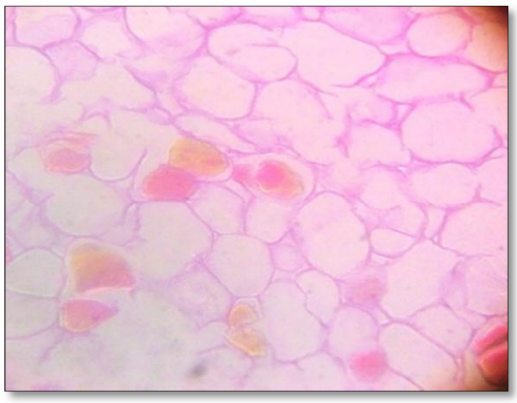
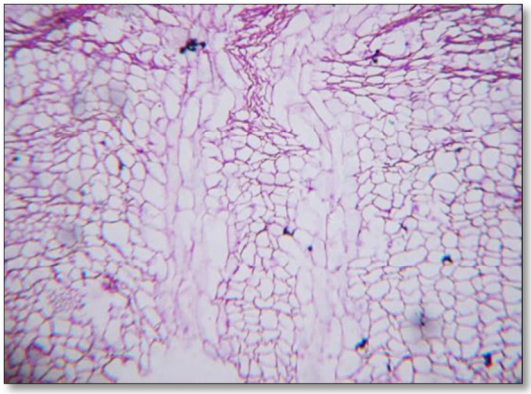


FIG NO: 9 CORTEX ENLARGED



FIGNO: 10 MEDULLARY RAYS ENLARGED

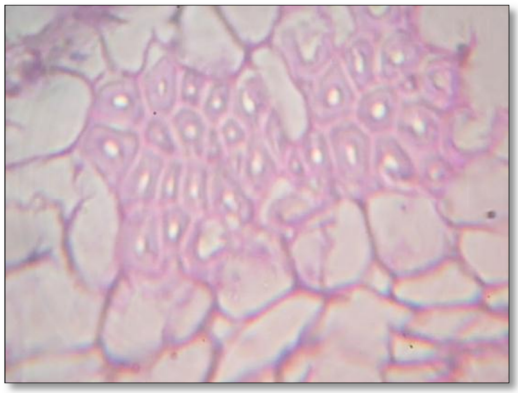


FIG NO: 11 PHLOEM FIBRES ENLARGED

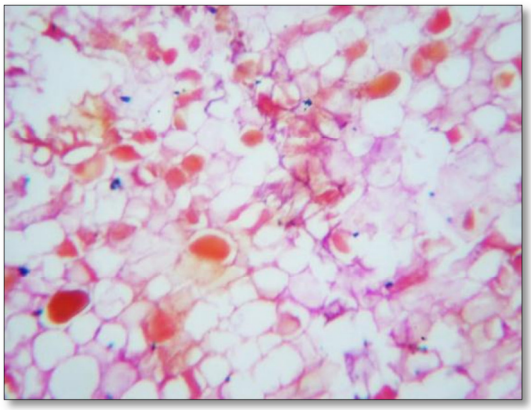


FIG NO: 12 SCLERENCHYMAENLARGED

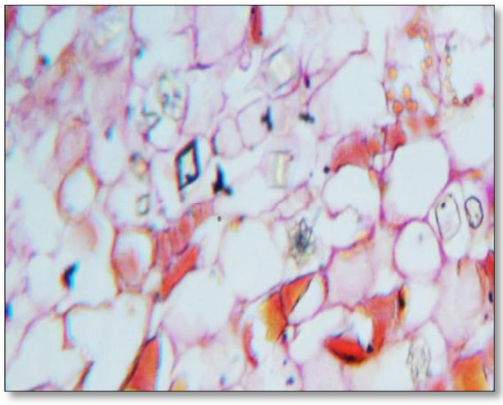


FIG NO: 13 CALCIUM OXALATE CRYSTALENLARGED

HISTOCHEMICAL COLOUR REACTIONS

Transverse section of bark of *Sterculia foetida* Linn., was treated with various reagents like Dragendroff's reagent, N/50 iodine, phloroglucinol and Conc. HCl, ferric chloride, picric acid and toludine blue. The histochemical color rections of transeverse section of bark showed different stainined cells were observed. These characterisitic features were observed and reported in the table no:3

Table no: 3 Histochemical colour reactions of *Sterculia foetida* Linn.,

S. No	Chemicals	Test for	Nature of change	Histology	Degree of change
1	**Phloroglucinol + HCl**	Lignin	Dark yellow	In the cortex region	+
2	**N / 50 Iodine solution**	Starch	Black	In the Cortex	+
3	**Dil. Ferric chloride**	Tannin	Bluish green	Phloem Parenchyma	+
4	**Dragendorff's Reagent**	Alkaloid	Brown	Outer cortex	+
5	**Picric acid**	Protein	Intense yellow	Phloem	+
6	**Toluidine blue**	Flavonoids	Bluish green	Cortex region	+

Note: + - Indicates the presence and absence

The histochemical analysis on the bark of *Sterculia foetida* Linn., showed the presence of alkaloids, starch grains, lignin, flavonoids are localized in the cortex region, proteins and tannins are localized in phloem region.

HISTOCHEMICAL STUDIES

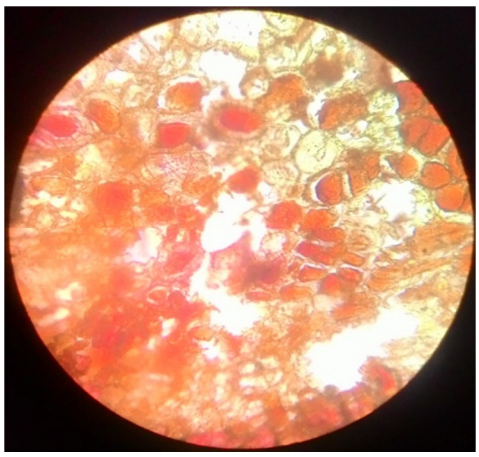


FIG NO: 14 Localization of Lignin (Pink) in cortex

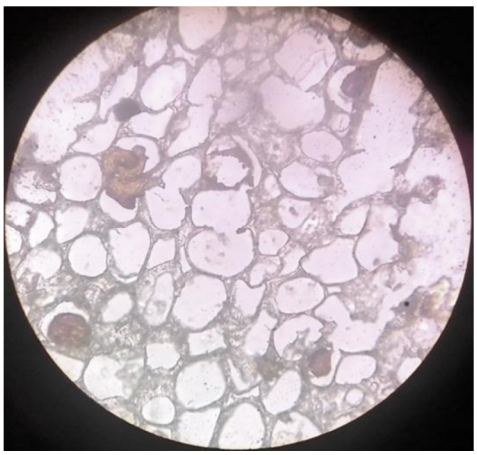


FIG NO: 15 Localization of Starch grains (Black) in cortex

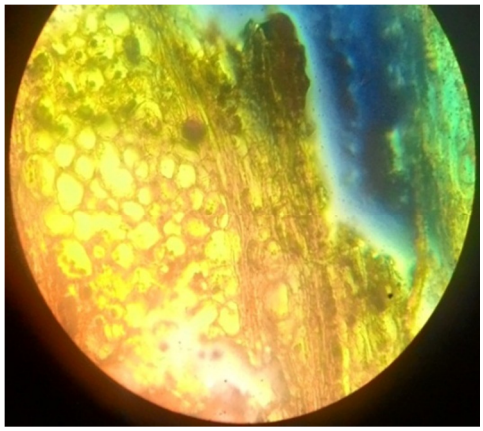


FIG NO:16 Localization of Tannins (Bluish green) in phloem parenchyma

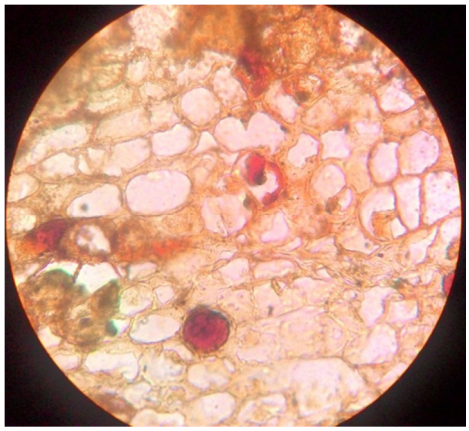


FIG NO:17 Localization of Alkaloid (Brown) in outer cortex

FIG NO:18 Localization of Protein (Intense yellow) in phloem cells

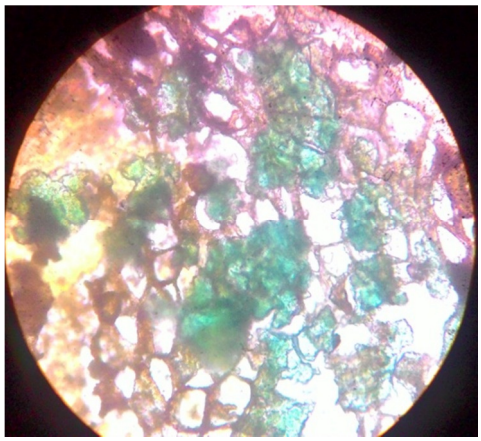


FIG NO:19 Localization of Flavonoid (Bluish green) in cortex

POWDER MICROSCOPY

ORGANOLEPTIC CHARACTERS

Nature	:	Coarse powder
Colour	:	Buff colour
Odour	:	Characteristic odour
Taste	:	Bitter taste

The powdered bark of *Sterculia foetida* under microscopic investigation showed the presence of lignified fibres, xylem, sclereids, phloem fibres, parenchymatous cells and calcium oxalate crystals.

Fibres

Fibres are lignified and scattered.

Phloem fibres

Phloem fibres occur lengthwise in groups of 3-5 cells, the brown masses are adhering to the fibres.

Calcium oxalate crystals.

Prismatic and rosette type calcium oxalate crystals in the cortical parenchyma and as well free in the powder are seen.

Cork

Occasional fragments of cork with thin walled cells, polygonal surface view and containing brownish matter.

Sclereids (or) stone cells

Scleredis which are ovoid to irregular in shape and yellowish in colour, (appears as 'U' shaped as one wall is thick than other).

POWDER MICROSCOPY

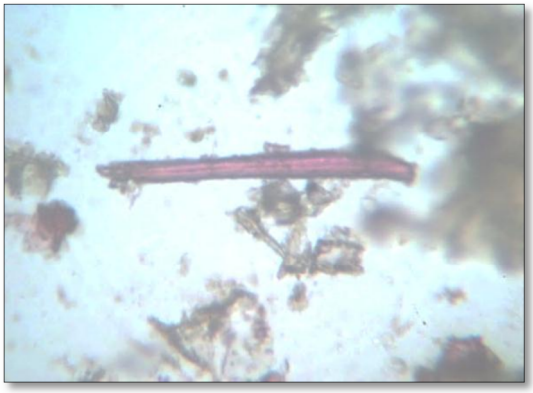


FIG NO: 20 FIBERS

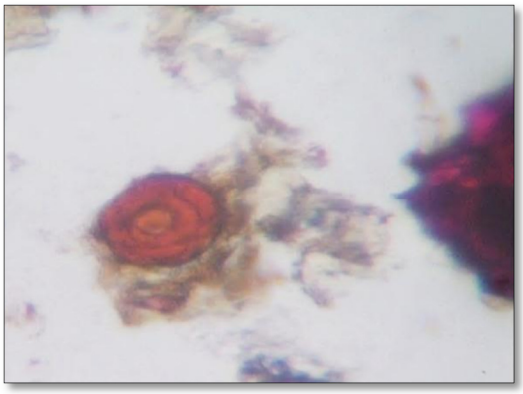


FIG NO: 21 SCLEREIDS

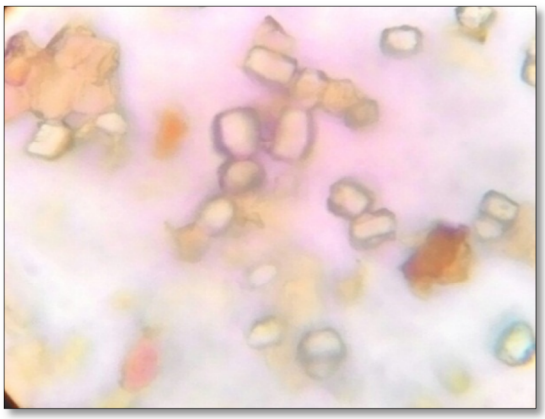


FIG NO: 22 CALCIUM OXALATE CRYSTALS

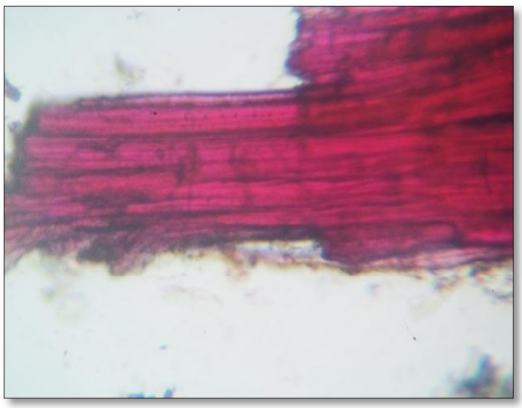


FIG NO: 23 PHLOEM FIBRES

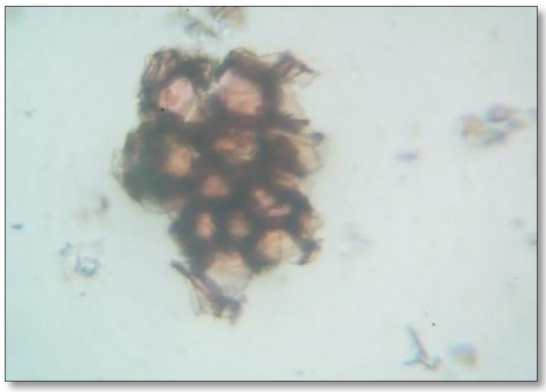


FIG NO: 24 CORK

QUANTITATIVE MICROSCOPY

Linear measurement of fibres

The length and width of the fibres were measured in the powdered bark of *Sterculia foetida* Linn., and the results were shown in table no: 4.

TABLE NO: 4 LINEAR MEASUREMENT OF FIBERS

Dimension	Minimum (µm)	Average (µm)	Maximum (µm)
Length	365	423	531.2
Width	49.8	54.78	66.4

The length of the fibers were in the range of 365µm - 423 µm and the width of the fibers was found in the range of 49.8 µm – 54.78 µm.

PHYSIOHEMICAL CONSTANTS

Physiochemical constants like total ash values, acid insoluble ash, water soluble ash, extractive values, loss on drying, swelling index, foaming index, foreign organic matter were studied and reported in table no: 5.

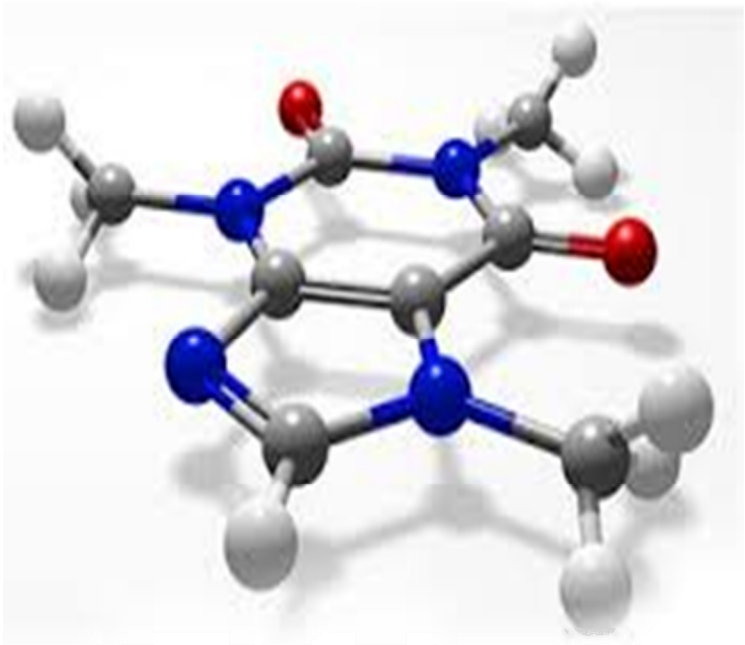
TABLE NO: 5 PHYSIOHEMICAL ANALYSIS OF THE BARK OF *Sterculia foetida* Linn.,

S.NO	PHYSIO-CHEMICAL CONSTANTS	RESULTS (%W/W)
I	**ASH VALUE**	
1.	Total ash	18.50±0.16
2.	Water soluble ash	8.26±0.75
3.	Acid insoluble ash	3.69±0.33
4.	Sulphated ash	11.68±0.36
II	**EXTRACTIVE VALUE**	
1.	Water soluble extractive	3.56±0.42
2.	Alcohol soluble extractive	6.89±0.53
3.	Ether soluble extractive	5.63±0.21
4.	Non-volatile ether soluble extractive	4.64±0.52
III	**Loss on drying**	1.88±0.50
IV	**Foaming index**	Nil
V	**Swelling index**	Nil
VI	**Foreign organic matter**	0.96±0.54

Values are expressed as Mean ± SD, n=3

The foreign organic matter of bark of *Sterculia foetida* Linn., was found to be 0.96±0.54% w/w. The total ash, acid insoluble ash, water soluble ash and sulphated ash were found to be 18.50±0.16% w/w, 3.69±0.33% w/w, 8.26±0.75% w/w and 11.68±0.36% w/w respectively. The water soluble extractive, alcohol soluble extractive, ether soluble extractive, and non-volatile extractive values were found to be 3.56±0.42% w/w, 6.89±0.53% w/w, 5.63±0.21% w/w, 4.64±0.52% w/w respectively. Loss on drying was found to be 1.88±0.50% w/w. The foaming index and swelling index was found to be nil this indicates the absence of mucilage and saponins.

This detailed Pharmacognostical investigation on the bark of *Sterculia foetida* Linn., will be useful for the identification of the drug in crude as well as powder form in order to differentiate the plant from its allied species and adulterants.



Phytochemical studies

7. PHYTOCHEMICAL STUDIES

7.1. MATERIAL AND METHODS

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant by using suitable chemical tests. Phytochemical analysis is very much important because the therapeutic activity is based on the constituent present in the drug. It can be done by confirmation with different chromatographic techniques like TLC and HPTLC. Therefore a complete investigation is required to characterize the Phytoconstituents qualitatively and quantitatively.

PREPARATION OF EXTRACTS

Fresh bark was collected, dried in shade, coarsely powdered and successively extracted with solvents of increasing polarity like n-hexane, chloroform, ethyl acetate and ethanol by continuous percolation process using soxhlet apparatus. After extraction each extracts were concentrated by using rotary vacuum evaporator. It is dried and the percentage yield was calculated. Appearance and consistency of the extract were also noted.

PRELIMINARY PHYTOCHEMICAL SCREENING[46-48]

The bark powder and extracts were subjected to qualitative chemical analysis for the identification of active constituents in each extracts and the powdered bark.

DETECTION OF ALKALOIDS

Dragendorff's reagent

To the sample 5ml of 2M HCl was added. Then 1ml of Dragendorff's reagent was added and examined for an immediate formation of an orange red precipitate.

Mayer's reagent

To the substance little quantity of dilute hydrochloric acid and Mayer's reagent were added and examined for the formation of white precipitate.

Wagner's reagent

The test substance was treated with little amount of Wagner's reagent and examined for the formation of reddish brown precipitate.

DETECTION OF GLYCOSIDES

Borntrager's test

The powdered material was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer indicates the presence of anthroquinone glycosides.

Modified Borntrager's test

The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

Legal's test

The test sample when treated with sodium nitropruside in pyridine and methanolic alkali. Formation of a pink red colour indicates the presence of cardiac glycosides.

DETECTION OF STEROIDS AND TRITERPENOIDS

LibermannBurchards Test

The powdered drug was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube formation of brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red color indicates presence of tri terpenoids.

Salkowski Test

The extract was treated with few drops of concentrated sulphuric acid, red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of tri terpenoids.

DETECTION OF FLAVONOIDS

Shinoda's test

Small quantity of extract was dissolved in alcohol to this pieces to magnesium followed by concentrated hydrochloric acid were added drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

Alkaline reagent test

Small quantity of the extract was dissolved in aqueous sodium hydroxide and appearance of yellow colour indicates the presence of flavonoids.

DETECTION OF CARBOHYDRATES

Molisch's test

To the test solution few drops of alcoholic alpha napthol and few drops of conc. sulphuric acid were added through the sides of test tube, purple to violet color ring appears at the junction indicates the presence of carbohydrates.

Fehling's test

The test solution was mixed with Fehling's I and II, heated and examined for the appearance of red coloration for the presence of sugar.

DETECTION OF TANNINS

Lead acetate test

The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.

Ferric chloride test

A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black color.

DETECTION OF PROTEINS

Biuret test

The sample was treated with 5-8 drops of 10% w/w copper sulphate solution and observed for the presence of violet color.

DETECTION OF SAPONINS

A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3 minutes. Development of any honey comb like froth was examined.

DECTECTION OF GUMS AND MUCILAGE

The small quantities of test substance were dissolved in 5 to 10ml of acetic anhydride by means of heat, cooled and added 0.05ml of conc. sulphuric acid. Formation of bright purplish red color indicates the presence of gums and mucilage.

DETECTION OF FIXED OILS AND FATS

Small quantities of extracts were pressed between two filter papers. An oily stain on the filter paper indicates the presence of fixed oils and fats.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

ESTIMATION OF ALKALOID CONTENT[49-51]

Mobile phase

About 9.93 g of monobasic potassium phosphate was dissolved in 730 mL of distilled water. Added 270 mL of acetonitrile, mixed, filtered, and degased.

Standard solution

About 0.2mg of standard is accurately weighed and dissolved in a mixture of water and methanol in the ratio of 1 : 1.

Test solution

1gm of finely powder sample was transferred to a continuous-extraction apparatus with a 500-mL round-bottom flask. Treated with 150 mL of methanol and extracted for 6 hours, or until the solvent is clear. The volume of the thimble should be at least one-half that of the volume of methanol. Cooled to room temperature, and transferred the methanol extract to a 200-mL volumetric flask. Rinsed the extract unit with methanol, quantitatively transferred the contents to the volumetric flask and diluted with methanol to volume.

Method

This HPLC method was applied in the validation of the ambient extraction method.

Chromatographic system

The liquid chromatography is equipped with a 235-nm detector and a 4.6-mm ×150-mm column that contains packing L1.The flow rate is about 1.8 mL per minute.

Procedure

Separately equal volumes (about 10 µL) of the Standard solution and the Test solution was injected into the chromatograph, recorded the chromatograms, and areas of the major peaks was measured. Calculate the percentage of alkaloids present in the solution using the formula.

100(CV/W) (r U / r S),

Where,

C - the concentration in mg per mL, Reference Standard in the Standard solution using the correction factors as noted above,

V - the final volume in mL of the Test solution,

W - the weight in mg

rU and rS are the peak areas from the Test solution and the Standard solution.

ESTIMATION OF FLAVONOID CONTENT[52-54]

Extraction solvent— mixture of alcohol, water and hydrochloric acid (50:20:8).

Mobile phase

Mixture of methanol, water, and phosphoric acid (100:100:1).

Standard solutions

Transfer accurately weighed quantities of USP Quercetin RS, kaempferol and iso-rhamnetin to separate volumetric flasks, dissolve each in methanol, and diluted quantitatively and stepwise if necessary, with methanol to obtain Standard solutions 1 mg per mL, respectively.

Test solution

Accurately weighed about 10.0 g of sample was transferred to a 250-mL flask fitted with a reflux condenser. Added 78 mL of Extraction solvent, and reflux on a hot water bath for 135 minutes cooled at room temperature and decanted to a 100-mL volumetric flask. 20 mL of methanol was added and sonicated for 30 minutes. Filtered and collected the filtrate in the 100-mL volumetric flask washed the residue on the filter with methanol and collected the washing in the same 100-mL volumetric flask.

Chromatographic system

The liquid chromatography is equipped with a 270-nm detector and a 4.6-mm × 25-cm column that contains packing L1. The flow rate is about 1.5 mL per minute.

Procedure

Separately injected equal volumes (about 20 µL) of each of the Standard solutions and the test solution into the chromatography, record the chromatograms and measured the areas for the major peaks. Calculated the percentage of flavonoid present in each sample.

ESTIMATION OF STEROIDS[55]

Standard solution

Weighed accurately a suitable quantity of the reference substance specified in the individual monograph, previously dried under the conditions specified in the monograph and dissolve in a suitable volume of aldehyde - free ethanol. Dilute quantitatively and stepwise with aldehyde - free ethanol to obtain a solution containing about 10g of the steroid per ml.

Test Solution

It is prepared by dissolving in a extraction solvent and made up to the volume in a standard flask.

Method

Into a glass - stoppered, 50 ml, conical flask and 20.0 ml of the test solution was taken. Into two similar flasks pipetted 20.0 ml of the standard solution and 20.0 ml of aldehyde - free ethanol (Blank) respectively. To each flask 2.0 ml of blue

tetrazolium solution was added and mixed to each flask add 2.0 ml of the mixture of 10 volumes of tetramethylammonium hydroxide solution (10%) and 90 volumes of aldehyde - free ethanol was added, mixed and allowed to stand in the dark a temperature between 25 and 35. At the end of exactly 90 minutes add to each flask 1.0 ml of glacial acetic acid was added and mixed. Measure the absorbance's of the solutions obtained from the test solution and the standard solution at about 525 nm against the blank. The quantity in mg, of the steroid in the 20-ml aliquot of the test solution is given by the expression

At / As X Cs

Where,

At = absorbance of the test solution

As = absorbance of the standard solution

Cs = quantity in mg, of the reference substance in the 20-ml aliquot of the standard solution.

The quantity of the steroid in the substance being examined was calculated on the basis of the aliquot the test solution taken for the determination and from the declared content of the steroid in the appropriate reference substance.

FLUORESCENCE ANALYSIS[56, 57]

Many crude drug show Fluorescence when the sample is exposed to UV radiation. Evaluation of crude drugs based on fluorescence in day light is not much used, as it is usually unreliable due to the weakness of the fluorescent effect. Fluorescent lamps are fitted with suitable filter, which eliminate visible radiation from the lamp and transmits UV radiation of definite wavelength was used for the study several crude drugs show characteristic fluorescence which is very much useful for their evaluation.

CHROMATOGRAPHY

Chromatographic methods are important analytical tool for the separation and identification of active components present in the plant.

THIN LAYER CHROMATOGRAPHY[58]

Principle

The principle of separation is adsorption. It is reliable technique in which solute undergoes distribution between two phases, stationary and mobile phase. The mobile phase flows through because of capillary action (against gravitational force). The compounds having higher affinities towards the stationary phase eluted slower were as the compound having lesser affinities towards stationary phase eluted faster.

TLC Plate Preparation

The plates were prepared using Stahl TLC spreader. 40gm of silica gel G was mixed with 85ml of water to prepare homogenous suspension and poured in the spreader. 0.25mm thickness of plates was prepared, air dried until the transparency of the layer disappeared, then dried at 110ºC for 30 minutes and kept in desiccators.

Selection of mobile phase

Solvent mixture was selected on the basis of the phytoconstituents present in each extract. Factors such as nature of components, stationary phase, polarity, influence the rate of separation of constituents was considered. From the vast analysis, best solvents were selected which showed good separation with maximum number of components.

$$R_f \text{ values} = \frac{\text{Distance travelled by solute from the baseline}}{\text{Distance travelled by solvent from the baseline}}$$

HPTLC- FINGERPRINT PROFILE[59]

HPTLC is one of the versatile chromatographic method which helps in the identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi- quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

HPTLC serves as a convenient tool for finding the distribution pattern of phytoconstituents which is unique to each plant. The fingerprint obtained was suitable for monitoring the identity and purity of drugs and for the detection of adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and to standardize the quantity of active principles in the herbal extract.

Instrument Conditions:

Sample used	**:**	Ethanol Extract of bark of *Sterculia foetida* Linn.,
Instrument	**:**	CAMAG HPTLC
Stationary phase	**:**	Aluminum coated silica Gel-Merk F254
Volume of injection	**:**	20µl
Mobile phase	**:**	Ethyl acetate : Toluene : Methanol : Formic acid (6:2:1:1)
Derivatizing agent	**:**	10% Anisaldehyde in sulphuric acid
Lamp	**:**	Tungsten
Lambda max	**:**	400-800nm

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 12 × 3 cm (H x W) pre-activated HPTLC silica Gel Merk F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator 8mm from the bottom.

7.2. RESULTS AND DISCUSSION

Phytochemical investigations were carried and the results are as follows.

Table no: 6 Percentage yield of successive extracts of bark of *Sterculia foetida* Linn.,

S.NO	EXTRACT	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	YIELD (%W/W)
1.	**n-Hexane**	Continuous percolation method using Soxhlet apparatus	Solid	Buff colour	1.64
2.	**Chloroform**		Semisolid	Brown colour	1.28
3.	**Ethyl acetate**		Semisolid	Brown colour	1.08
4.	**Ethanol**		Solid	Brown colour	2.26

QUALITATIVE PHYTOCHEMICAL ANALYSIS

TABLE NO: 7 PRELIMINARY PHYTOCHEMICAL ANALYSIS

S.NO	CHEMICAL CONSTITUENTS	POWDERED DRUG	n-HEXANE EXTRACT	CHLOROFORM EXTRACT	ETHYL ACETATE EXTRACT	ETHANOL EXTRACT
1.	**Steroids**	+	+	+	+	+
2.	**Glycosides**	-	-	-	-	-
3.	**Saponins**	-	-	-	-	-
4.	**Flavonoids**	+	-	+	+	+
5.	**Tannins**	+	-	-	+	+
6.	**Triterpenes**	+	+	+	+	+
7.	**Proteins**	+	-	-	-	+
8.	**Alkaloids**	+	-	+	+	+
9	**Carbohydrates**	+	-	+	+	+
10.	**Fats and oils**	-	-	-	-	-

Note: + - Indicates the presence and absence

From the qualitative analysis, it was observed that the ethanol extract showed the presence of maximum active constituents such as flavonoids, tannins, triterpenes, proteins and alkaloids. All these compounds were found in the powdered bark also. The ethyl acetate extract also showed the presence of more number of active constituents. Chloroform extract showed the presence of flavonoids, teriterpenes and alkaloids. n-Hexane extract showed the presence of steroids and teriterpenes.

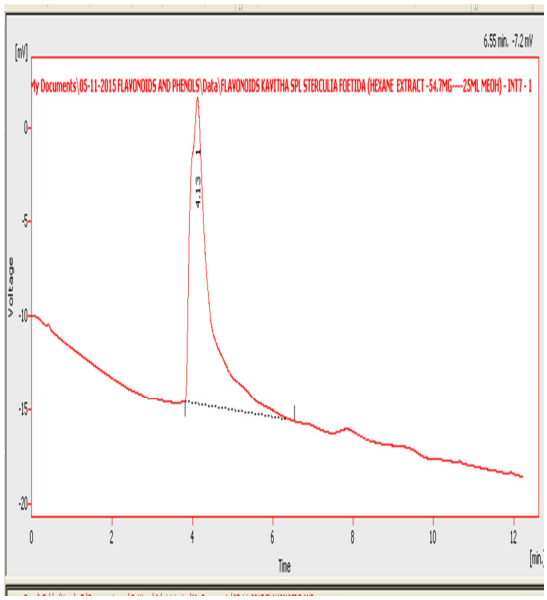
QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

The phytoconstituents like alkaloids, steroids, phenolic compounds and flavonoids were estimated quantitatively by HPLC method. The results were shown in the table no: 8.

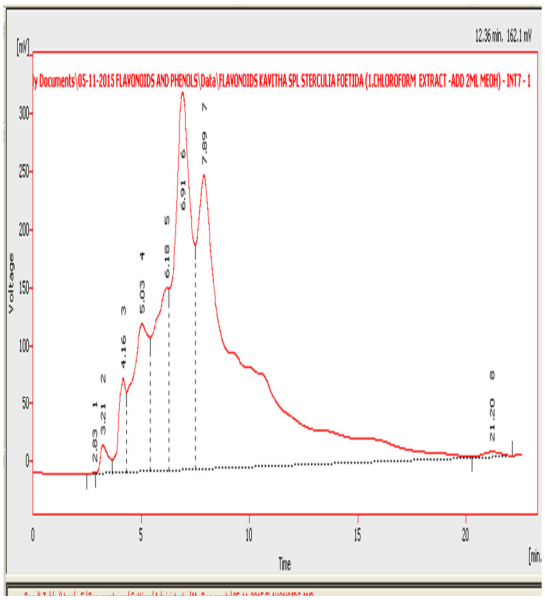
TABLE NO: 8 QUANTITATIVE ESTIMATION OF ALL THE EXTRACTS

S.No	Parameters	n-Hexane (mg/ml)	Chloroform (mg/ml)	Ethyl acetate (mg/ml)	Ethanol (mg/ml)
1.	Total Alkaloids	-	3.56	5.11	6.33
2.	Total steroids	6.09	2.09	3.02	4.11
3.	Flavonoids				
	Quercetin	1.03	0.09	0.08	0.09
	Rutin	-	0.81	0.813	0.413
	Kamferol	-	0.095	-	0.396
	Thymoquine	-	0.645	0.041	0.263
	Gallagin	-	0.034	-	0.044
4.	Phenolic compounds				
	Ascorbic acid	0.92	0.53	0.65	1.34
	Tannic acid	-	1.45	0.98	0.98
	Gallic acid	-	0.545	0.74	2.45
	Coumaric acid	-	0.84	0.094	1.35

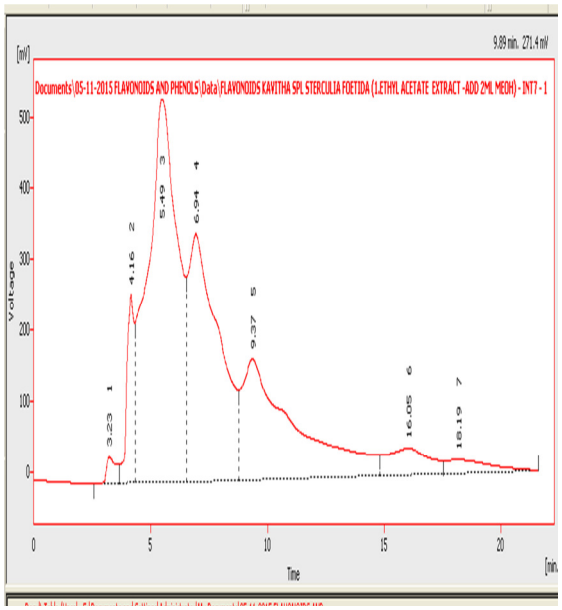
FIG NO: 24 ESTIMATION OF FLAVONOID CONTENT BY HPLC METHOD



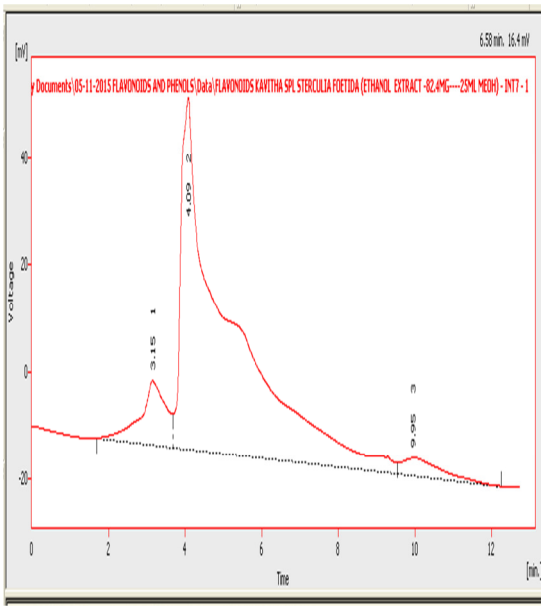
n-HEXANE EXTRACT OF *Stercuia foetida* Linn.,



CHLOROFORM EXTRACT OF *Stercuia foetida* Linn.,

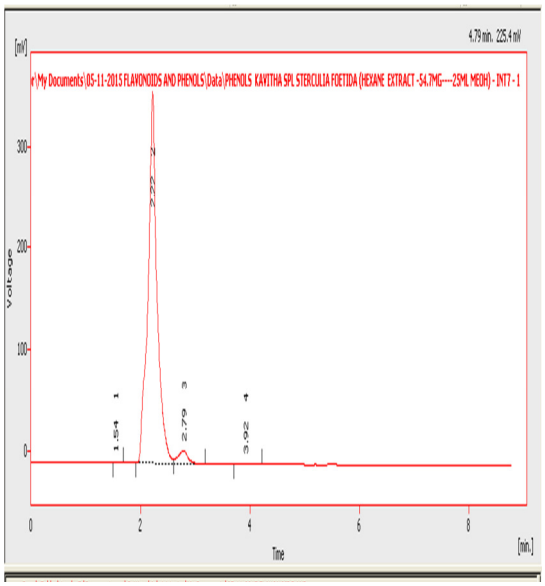


ETHYL ACETATE EXTRACT OF *Sterculia foetida* Linn.,



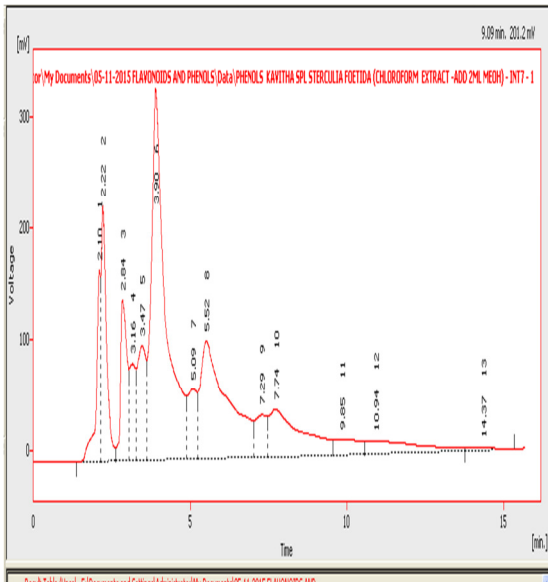
ETHANOL EXTRACTOF *Sterculia foetida* Linn.,

FIG NO: 25 ESTIMATION OF PHENOLIC CONTENT BY HPLC METHOD



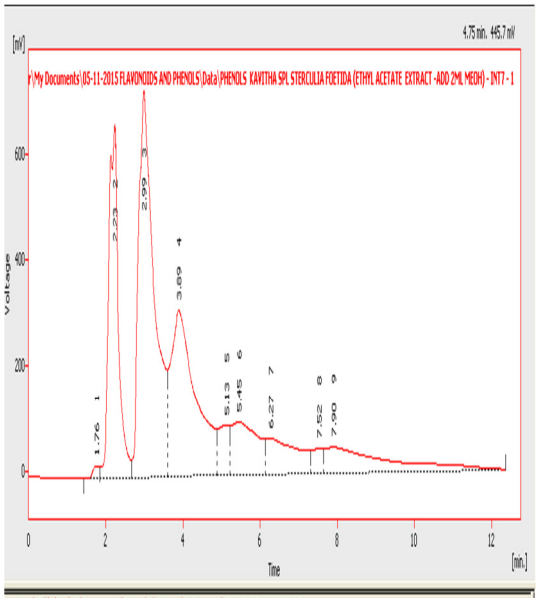
n-HEXANE EXTRACT OF

***Sterculia foetida* Linn.,**



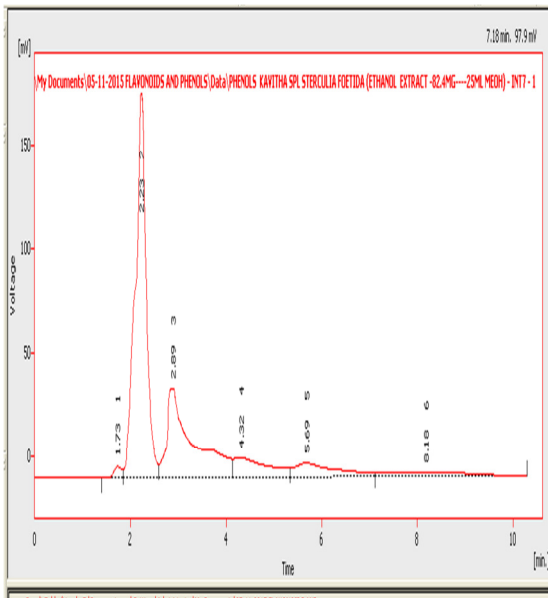
CHLOROFORM EXTRACT OF

***Sterculia foetida* Linn.,**



ETHYL ACETATE EXTRACTOF

***Sterculia foetida* Linn.,**



ETHANOL EXTRACT OF

***Sterculia foetida* Linn.,**

FLUORESCENCE ANALYSIS

TABLE NO: 9 FLUORESCENCE CHARACTERISTIC OF POWDERED BARK OF *Sterculia foetida* Linn.,

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	Powder	Orange brown	Blackish Brown	Orange
2.	Powder + water	Orange brown	Blackish Brown	Orange
3.	Powder + NaoH	Brown	Dark brown	Reddish brown
4.	Powder + HCl	Pale orange	Black	Light orange
5.	Powder + Acetic acid	Yellow	Black	Orange
6.	Powder + Alc.NaoH	Brown	Dark brown	Reddish brown
7.	Powder + Picric acid	Yellow	Brown	orange
8.	Powder + Sulphuric acid	Black	Black	Black
9.	Powder + Nitric acid	Reddish brown	Black	Red
10.	Powder + Iodine	Brown	Dark brown	Orangish brown
11.	Powder + $Fecl_3$	Green	Dark green	Light green
12.	Powder + KOH	Brown	Black	Reddish orange
13.	Powder + alc.KOH	Brown	Black	Reddish orange
14.	Powder + ammonia	Light brown	Black	Orange
15.	Powder + ethanol	Light brown	Dark brown	Orange

TABLE NO: 10 FLUORESCENCE ANALYSIS OF VARIOUS BARK EXTRACTS OF *Sterculia foetida* Linn.,

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	**n-Hexane**	Buff colour	Pale brown	Buff colour
2.	**Chloroform**	Dark red	Black	Brown
3.	**Ethyl acetate**	Dark red	Black	Brown
4.	**Ethanol**	Dark red	Black	Brown

There was no characteristic fluorescence were seen with either the powdered bark or the extracts.

CHROMATOGRAPHIC STUDIES

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was done with all the four extracts and their R_f values were tabulated in the table no: 11.

TABLE NO: 11 THIN LAYER CHROMATOGRAPHIC STUDIES OF EXTRACT

S.NO	EXTRACTS	SOLVENT SYSTEM	NO. OF SPOTS	R_F VALUES
1.	**n-Hexane**	Toluene : methanol (9:1)	1	0.7
2.	**Chloroform**	Toluene : methanol (9:1)	2	0.28, 0.56
3.	**Ethyl acetate**	Ethyl acetate : acetonitrile (3:2)	2	0.31,0.45
4.	**Ethanol**	Ethyl acetate : Toluene : Methanol : Formic acid (6:2:1:1)	4	0.33,0.45.0.57,0.71

HPTLC FINGER PRINT PROFILE

HPTLC Finger print Data of Ethanolic Extract of *Sterculia foetida* Linn.,

Since the yield of ethanolic extract was high and it was found to contain more phytoconstituents it was selected for the HPTLC studies. High performance thin layer chromatography (HPTLC) finger printing was performed with the ethanol extract of *Sterculia foetida* Linn.,

FIG NO: 26 HPTLC OF ETHANOLIC EXTRACT

FIG NO: 27 HPTLC FINGER PRINT OF ETHANOLIC EXTRACT OF *Sterculia foetida* Linn.,

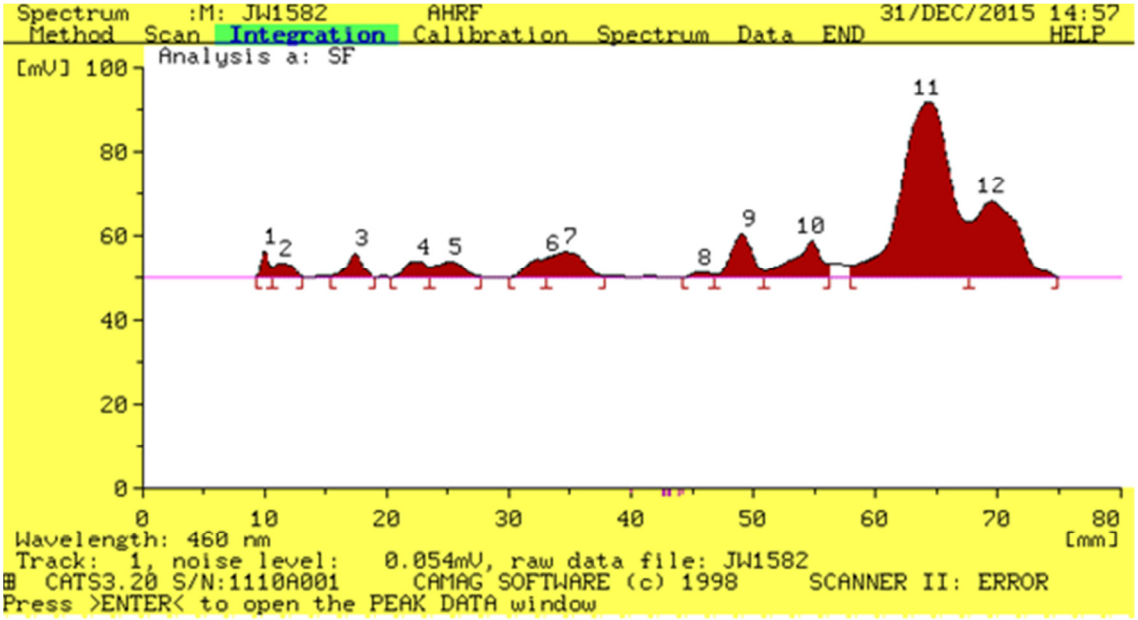
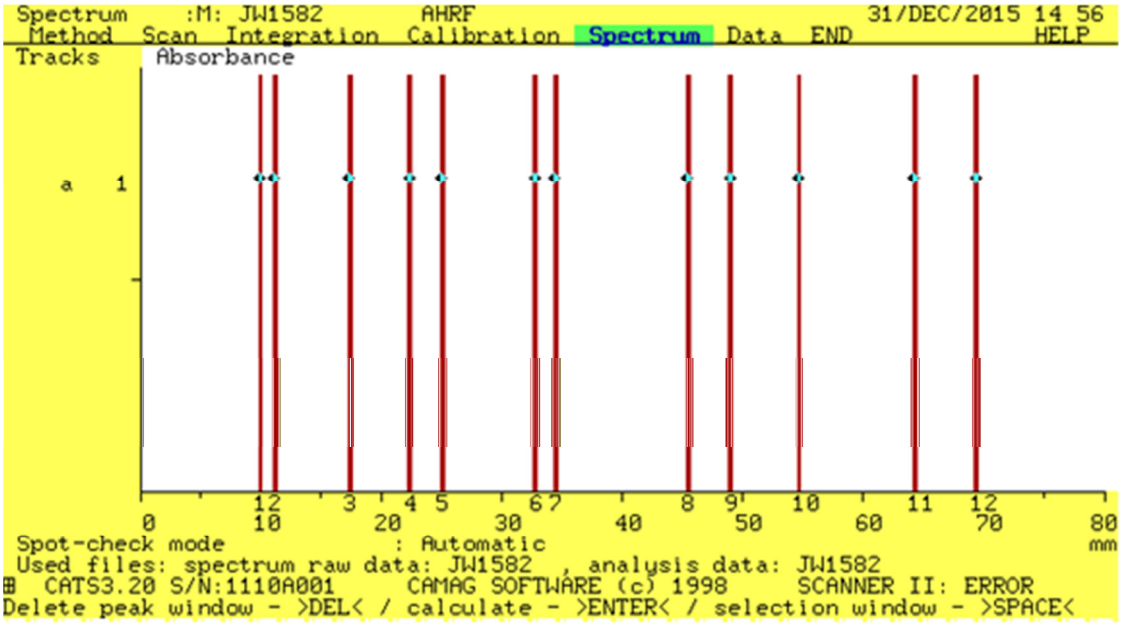


FIG NO: 28 HISTOGRAM OF ETHANOLIC EXTRACT OF BARK OF *Sterculia foetida* Linn.,



FINGER PRINT DATA ANALYSIS FOR HPTLC

TABLE NO: 12 HPTLC FINGER PRINT DATA

S. No	R_f	Height	Area	Lambda Max
1	0.10	6.3	44.1	800
2	0.11	3.3	62.3	710
3	0.17	5.8	86.6	710
4	0.22	4.0	80.6	710
5	0.25	3.8	100.6	710
6	0.33	4.6	81.9	710
7	0.34	6.1	191.9	710
8	0.45	1.5	24.7	710
9	0.49	10.3	206.6	710
10	0.54	8.6	236.2	710
11	0.64	41.9	1949.5	610
12	0.69	18.1	753.2	710

There were 12 peaks was observed with different R_f values and different percentage of areas. HPTLC was scanned at 460nm with the best solvent to detect the maximum number of components and peak abundance quantitatively. The finger print of ethanolic extract showed 12 peaks with different R_f values.

Since secondary metabolites are responsible for biological activity, this study would be the leading pathway of information for selection of extract for pharmacological activity and isolation of constituents responsible for the activity.

Selection of Active extract

9. SELECTION OF ACTIVE EXTRACT

9.1. MATERIALS AND METHODS

The plant *Sterculia foetida* Linn., contains many active constituents in each extracts. Hence for all the extracts in order to select the best extract *in-vitro* studies were carried out.

Hence all the extracts of bark of *Sterculia foetida* were subjected to *in-vitro* antioxidant activity and anti-arthritic activity studies. These studies are used for the selection of best active extract which would take to precede the further activities.

IN VITRO ANTIOXIDANT ACTIVITY[60]

Antioxidant is a molecule that inhibits oxidation of other molecule which produces free radicals. These radicals in turns produce chain reactions there by cause damage to the cells, resulting in development of various ailments. Antioxidants terminate these chain reactions by removing free radicals and inhibiting oxidative reactions. Therefore, antioxidant with free radical scavenging effect will be of greater importance in the prevention and therapeutics of disease.

DPPH ASSAY (2, 2-DIPHENYL -1-PICRYLHYDRAZYL)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Different volumes (1.25-10µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

$$\% \text{ Inhibition} = \frac{\text{Abs(control)} - \text{Abs(test)}}{\text{Abs(control)}} \times 100$$

NITRIC OXIDE SCAVENGING ACTIVITY

Nitric oxide scavenging activity of the extract was determined by using aqueous solution of sodium nitroprusside which generates nitric oxide (NO) spontaneously at physiological pH. NO interacts with oxygen to produce nitrate ions which were measured calorimetrically. 3 ml of reaction mixture containing 2 ml of sodium nitroprusside (10mM) in Phosphate buffer solution and 1 ml of various concentrations of successive extracts were incubated at 37°C for 4 hours. Control without test compound was kept in the same manner. After incubation 0.5 ml of Griess reagent (0.2% naphthylenediaminedihydrochloride, 2% sulphanilamide in 5% phosphoric acid) was added. The absorbance of the chromophore formed was read at 546 nm and compared with standard Ascorbic acid.

$$\% \text{ Inhibition} = \frac{\textbf{Abs(control) - Abs(test)}}{\textbf{Abs(control)}} \textbf{ X 100}$$

***IN-VITRO*ANTI-ARTHRITIC ACTIVITY[61]**

PROTEIN DENATURATION METHOD

The test and standard solutions were prepared and pH was adjusted to 6.3 to all the solution by using 1N HCl. All the sample solution was incubated at 37^{O}C for 20minutes and the temperature was increased to 57^{O}C for 3 minutes. Allowed to cool for some time then 2.5ml of Phosphate buffer was added to all the above solutions. The absorbance was measured at 416nm using UV visible spectrophotometer. The Percentage inhibition of protein denaturation was calculated.

$$\textbf{Protein denaturation assay} = \frac{\textbf{(O.D Test compound - O.D control)}}{\textbf{(O.D control)}} \textbf{ X 100}$$

RESULTS

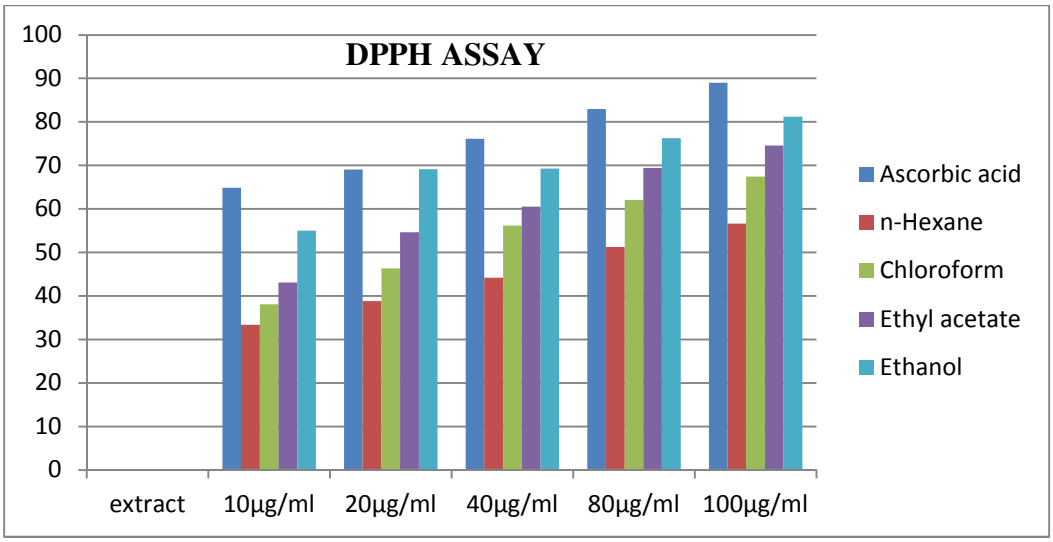
IN-VITRO ANTIOXIDANT ACTIVITY

DPPH ASSAY (2, 2-DIPHENYL -1-PICRYLHYDRAZYL)

TABLE NO: 13 PERCENTAGE INHIBITION OF EXTRACTS-DPPH ASSAY

Extract	% Inhibition at various concentrations				
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	100 µg/ml
Ascorbic acid	61.03	68.88	75.04	82.04	88.31
n-Hexane	34.75	41.28	47.96	53.91	58.76
Chloroform	35.20	46.07	53.24	59.77	62.08
Ethyl acetate	39.48	49.09	56.52	61.84	67.31
Ethanol	56.09	62.15	68.23	73.01	79.53

FIG NO: 29 GRAPHICAL DATA OF PERCENTAGE INHIBITION



In DPPH assay the scavenging potential of the antioxidant compounds or extracts were found. The percentage inhibition of n-Hexane, Chloroform, Ethyl acetate and Ethanol was found to be 58.76, 62.08, 67.31 and 79.53 respectively at a maximum concentration of 100µg/ml. The percentage inhibition of Ascorbic acid

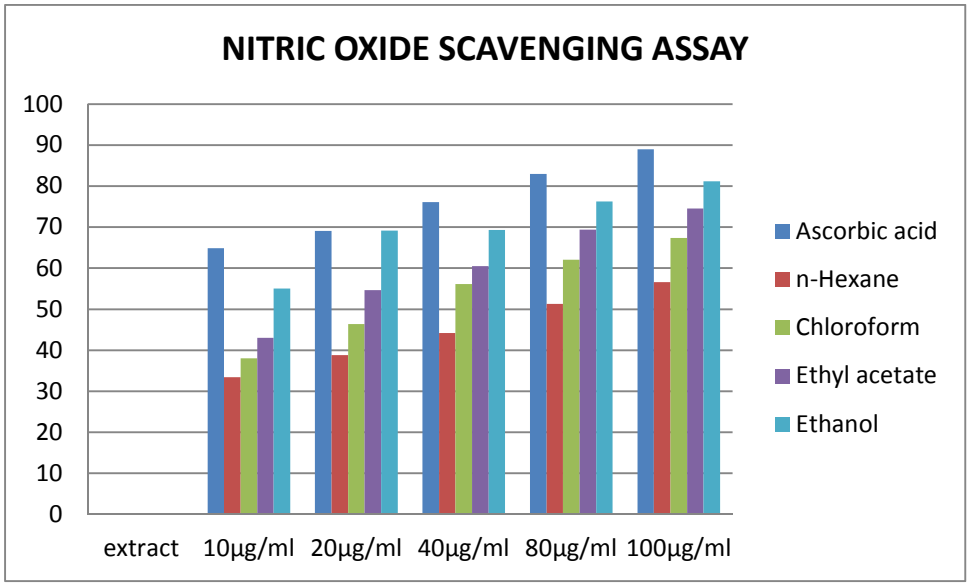
(Standard) was found to be 88.31. In that ethanolic extract showed the maximum percentage of inhibition which is comparable to the standard.

NITRIC OXIDE SCAVENGING ASSAY

TABLE NO: 14 PERCENTAGE INHIBITION OF EXTRACTS-NITRIC OXIDE SCAVENGING ASSAY

Extract	% Inhibition at various concentrations				
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	10 µg/ml
Ascorbic acid	64.85	69.08	76.10	82.99	89.01
n-Hexane	33.41	38.81	44.22	51.27	56.62
Chloroform	38.06	46.35	56.16	62.08	67.41
Ethyl acetate	43.06	54.63	60.54	69.40	74.58
Ethanol	55.04	69.14	69.29	76.29	81.19

FIG NO: 30 GRAPHICAL DATA OF PERCENTAGE INHIBITION



In nitric oxide scavenging assay the scavenging potential of all the extracts were found. The percentage inhibition of all the extracts was found to be 56.62, 67.41, 74.58 and 81.19 respectively at the maximum concentration of 100µg/ml. The

percentage inhibition of Ascorbic acid (Standard) was found to be 89.01. This result showed that ethanolic extract showed the maximum percentage inhibition and possess maximum scavenging activity which is compared with the standard.

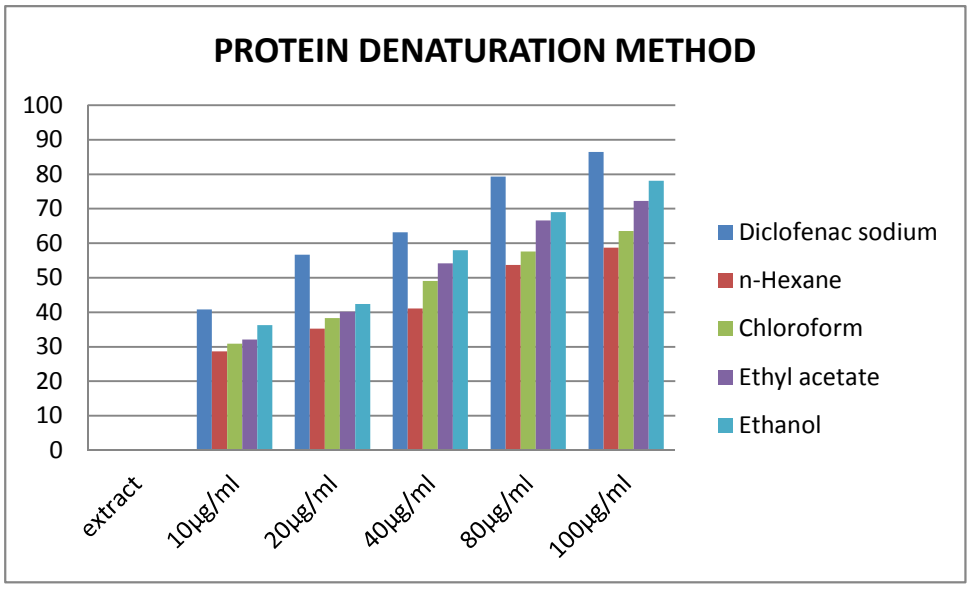
INVITRO ARTHRITIC ACTIVITY

PROTEIN DENATURATION METHOD

TABLE NO: 15 PERCENTAGE INHIBITION OF EXTRACTS – PROTEIN DENATURATION METHOD

Extract	% Inhibition at various concentrations				
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	10 µg/ml
Diclofenac sodium	40.85	56.63	63.20	79.31	86.41
n-Hexane	28.70	35.23	41.12	53.69	58.71
Chloroform	30.86	38.30	49.07	57.62	63.54
Ethyl acetate	32.12	40.13	54.18	66.58	72.30
Ethanol	36.27	42.36	57.96	69.03	78.09

FIG NO: 31 GRAPHICAL DATA OF PERCENTAGE INHIBITION



The percentage inhibition of protein denaturation assay by n-Hexane, Chloroform, Ethyl acetate and Ethanol was found to be 58.71, 63.54, 72.30 and 78.09 respectively at maximum concentration of 100µg/ml. Percentage inhibition of Diclofenac sodium (Standard) was found to be 86.41. The results indicated that ethanolic extract showed the maximum percentage inhibition which is compared with the standard.

Both the antioxidant studies and inhibition of protein denaturation assay indicated that of all the extracts, the ethanolic extract showed the maximum scavenging and inhibitory activity. This correlates with the findings of the phytochemical study where the ethanol extract showed the presence of most of the phytoconstituents.

Further studies are focused on *invivo* ant-arthritic activity of ethanolic extract and isolation of phytoconstituents which is responsible for the activity.

Pharmacological studies

10. PHARMACOLOGICAL STUDIES

10.1. ACUTE TOXICITY STUDY

10.1.1. MATERIALS AND METHOD

ACUTE TOXICITY STUDY (OECD 423 GUIDELINES)[62]

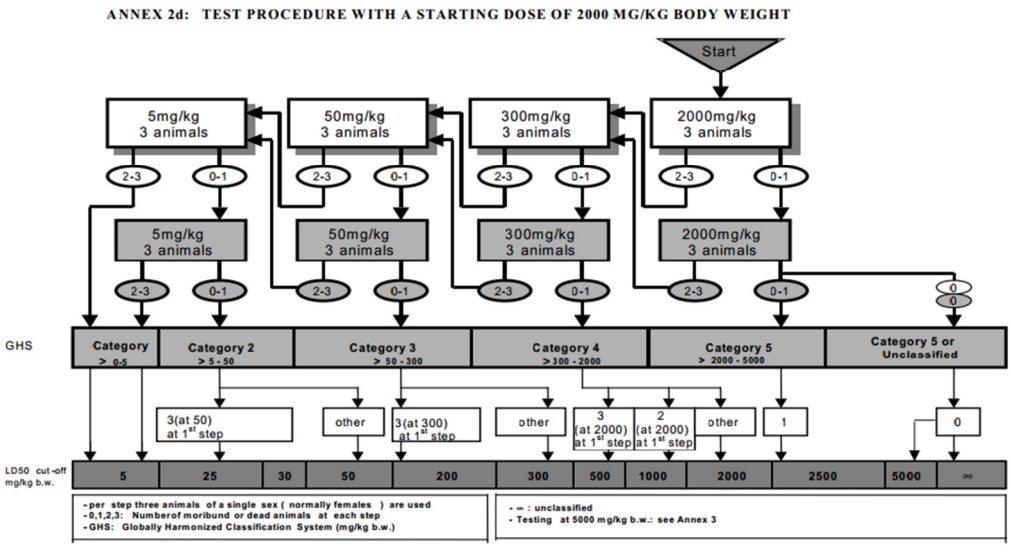
PROCEDURE: (Acute toxicity study is designed as per the OECD guidelines 423).

Adult Wistar albino rats of either sex weighing between 150-200g of 3 animals were selected for study. All the animals were fasted overnight provided with water ad libitum. Following period of fasting the test compound was administered at a dose of 2000mg/kg body weight orally.

Animals were observed individually after dosing periodically during the first 30 minutes to the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Observation includes changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, automatic, central nervous system, somato motor activated and behavioral pattern. Attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

The rats were observed regularly for 14 days to note the mortality or toxic symptoms. If no deaths were reported, the study was repeated with same dose to confirm the results.

FIG NO: 32 OECD Guidelines 423



10.1.2. RESULTS AND DISCUSSION

TABLE NO: 16 ACUTE TOXICITY-PARAMETERS STUDIED

OBSERVATION	30 mins	4 hrs	24 hrs	14th day
Body weight	-	-	-	-
Pre terminal deaths	-	-	-	-
Cage side observation	+	+	+	+
Motor activity	+	+	+	+
Convulsions	-	-	-	-
Pilorection	-	-	-	-
Righting reflex	+	+	+	+
Lacrimation	-	-	-	-
Salivation	-	-	-	-
Respiration	+	+	+	+
Skin color	+	+	+	+
Diarrhea	-	-	-	-
Loss of corneal reflex	-	-	-	-
Loss of pinnal reflex	-	-	-	-
Grooming	-	-	-	-
Sedation	-	-	-	-
Excitation	+	+	+	+
Aggression	+	+	+	+

NOTE: +, - Indicates presence or absence

Since there were no death and behavioral changes observed. The extract was considered as safe to administer. From this $1/10^{th}$ and $1/5^{th}$ of the dose (200mg and 400mg) were selected for further studies.

10.2. ANTI-ARTHRITIC ACTIVITY[63-69]

10.2.1. MATERIALS AND METHOD

ADJUVANT INDUCED ARTHRITIS MODEL

This is one of the most commonly used animal models for evaluating anti-arthritic activity. Arthritis is induced in rats by intra plantar injection of 0.1ml of Complete Freund's Adjuvant (CFA) in left hind paw except for the vehicle control. The adjuvant contained heat killed *mycobacterium tuberculosis*in sterile paraffin oil. The test drug and the standard drug will be administered orally for a period of 21 days and the paw volume was measured periodically using plethysmograph.

Test drug - Ethanolic extract were given at the dose of $1/10^{th}$ (200mg/kg) and $1/5^{th}$ (400mg/kg) for 21 days.

EXPERIMENTAL ANALYSIS

A total of 30 adult Wistar albino rats weighing (150-200g) were divided into 5 groups of 6 animals in each group.

TABLE NO: 17 GROUPING OF ANIMALS

S.NO	GROUP	NAME OF THE GROUP	TREATMENT
1	I	Positive control	Treatment with vehicle for 21 days
2	II	Arthritic control	Treated with CFA
3	III	CFA + Standard	Treatment with Diclofenac sodium 15mg/kg for 21 days
4	IV	CFA + Test drug1	Treatment with ethanol extract 200mg/kg for 21 days
5	V	CFA + Test drug2	Treatment with ethanol extract 400mg/kg 21 days

PARAMETERS STUDIED

CHANGES IN BODY WEIGHT

Body weight changes were observed every week.

PAW VOLUME MEASUREMENT

Paw volume of all the animal groups were measured by using plethysmograph at 0, 7, 14 and 21 days. The percentage inhibition of paw volume can be determined using this formula.

$$= \frac{(Vc - V0) - (Vt - V0) \times 100}{(Vc - V0)}$$

where, Vc - is the paw volume after induction

V0 - is the paw volume before induction

Vt - is the paw volume after treatment.

SECONDARY LESIONS

Secondary lesions developed in ears, fore limbs, hind limbs and tails were scored.

HEMATOLOGICAL PARAMETERS

At the end of the experimental period (22^{nd} day) the blood was collected from the animal through retro- orbital vein puncture of all the groups and the hematological parameters such as hemoglobin content, RBC count, WBC count, Hemoglobin and ESR were studied.

RADIOGRAPHICAL STUDIES

At the end of the experimental period, X- ray were taken for the hind limbs of experimental animals and examined for soft tissue swelling, bony erosions and narrowing of the spaces between the joints.

HISTOPATHOLOGICAL STUDIES

At the end of the experiment, animals were sacrificed by cervical decapitation for the evaluation of histopathological changes like soft tissue swelling, bone demineralization, pannus formation, cartilage erosion and joint space narrowing.

10.2.2. RESULTS AND DISCUSSION

IN-VIVO EVALUATION OFANTI-ARTHRITIC ACTIVITY BY ADJUVENT INDUCED ARTHRITIS (Complete Freund's Adjuvant)

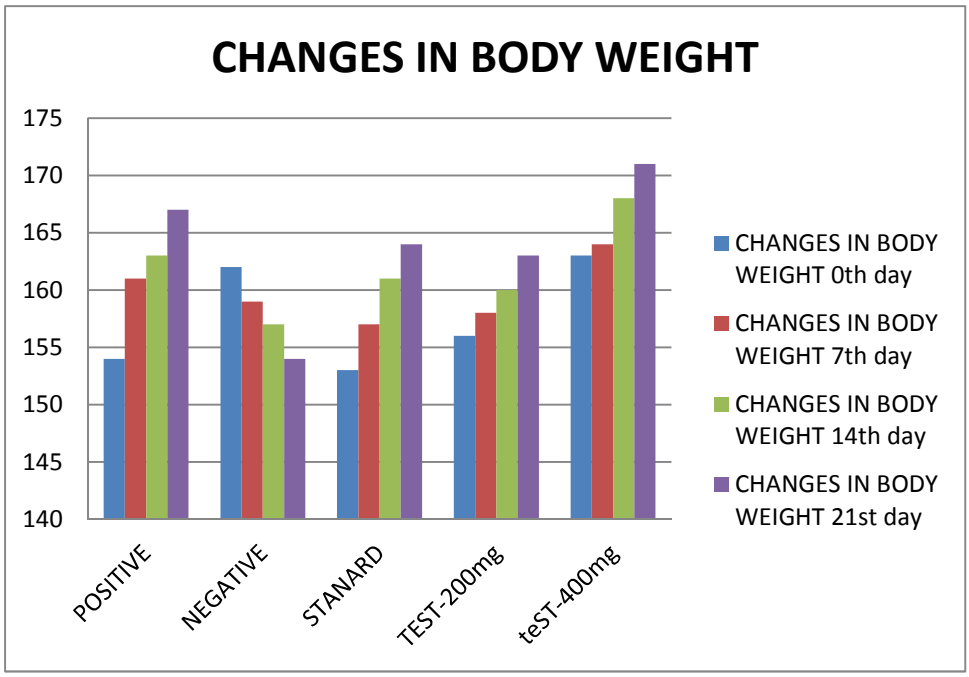
TABLE NO: 18 CHANGES IN THE BODY WEIGHT IN (GM) ADJUVANT-INDUCED ARTHRITIC RATS

Treatment	0^{th} day	7^{th} day	14^{th} day	21^{st} day
Group I	154 ±4.74	161 ± 9.58**	163 ± 9.3380**	167 ±10.30**
Group II (Arthritic control)	162 ± 6.408	159± 6.91*	157± 56.64*	154 ± 5.780*
Group III Diclofenac sodium	153 ± 8.846	157 ± 2.87***	161 ± 2.13***	164 ± 2.31b***
Group IV 200 mg/ kg	156 ± 4.49	158 ±5.09***	160 ±6.33***	163 ± 4.81***
Group V 400mg / kg	163 ± 5.79b	164 ±2.81***	168 ±4.979***	171 ± 5.42***

Values represented in the result are mean ± SD (n=6)

$^{*}p< 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to the positive and arthritis control. The data was analyzed using one way analysis of variance (ANOVA) followed by Tukey HSD Test.

FIG NO: 33 GRAPHICAL DATA OF CHANGES IN BODY WEIGHT-ADJUVANT INDUCED ARTHRITIC RATS



The body weight of the different groups of rats was noted. During the treatment the body weight of the control, standard, test dose I and test dose II animals were increased except arthritis induced rats.

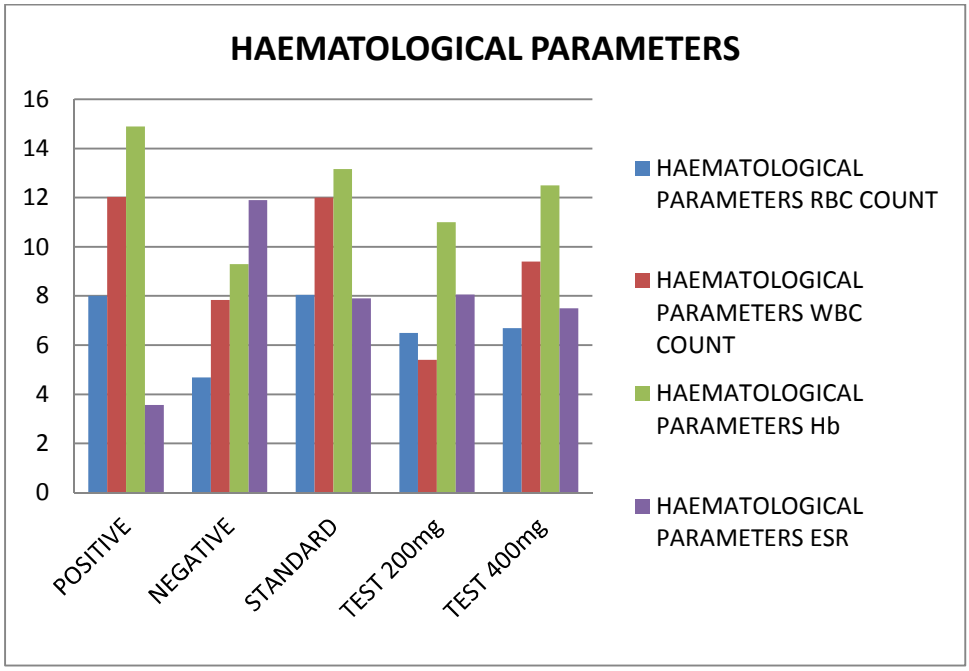
HAEMATOLOGICAL PARAMETERS

TABLE NO: 19 EFFECT OF HEMATOLOGICALPARAMETERS - ADJUVANT INDUCED ARTHRITIC RATS

PARAMETERS	RBC count (pre cu.mm)	WBC count (pre cu.mm)	Hb (g/dL)	ESR (mm/h.)
Group I (Control)	8.01±0.138	12.03± 1.15**	14.9±0.346**	3.56±0.342**
Group II (Arthritic control)	4.68±0.147	7.84±1.16*	9.3±0.257*	11.9±0.694*
Group III (Diclofenac sodium)	8.04± 0.264	12.0±0.41***	13.16±0.151***	7.9± 1.48***
Group IV (200 mg/ kg)	6.50±0.235	5.4±8.216***	11.0±0.258***	8.06±1.27***
Group V (400mg/kg)	6.69±0.102	9.4±0.755***	12.5±0.207***	7.5±0.843***

*p< 0.05, **p < 0.01, ***p < 0.001 as compared to the positive and arthritis control. The data was analyzed using one way analysis of variance (ANOVA) followed by Tukey HSD Test.

FIG NO: 34 GRAPHICAL DATA FOR EFFECT OF HEAMOTOLOGICAL PARAMETERS- ADJUVANT INDUCED ARTHRITIC RATS



The results indicate that arthritic group of animals showed decreased RBC, WBC and Hemoglobin levels. But the ESR level showed the increased value which is compared with the control group. In the standard group all the blood parameters are brought back to the normal levels. In the group which is treated with 200mg/kg showed the improvement of blood parameters and the extract of 400mg/kg showed significant improvement in blood parameters than the 200mg/kg treated animals.

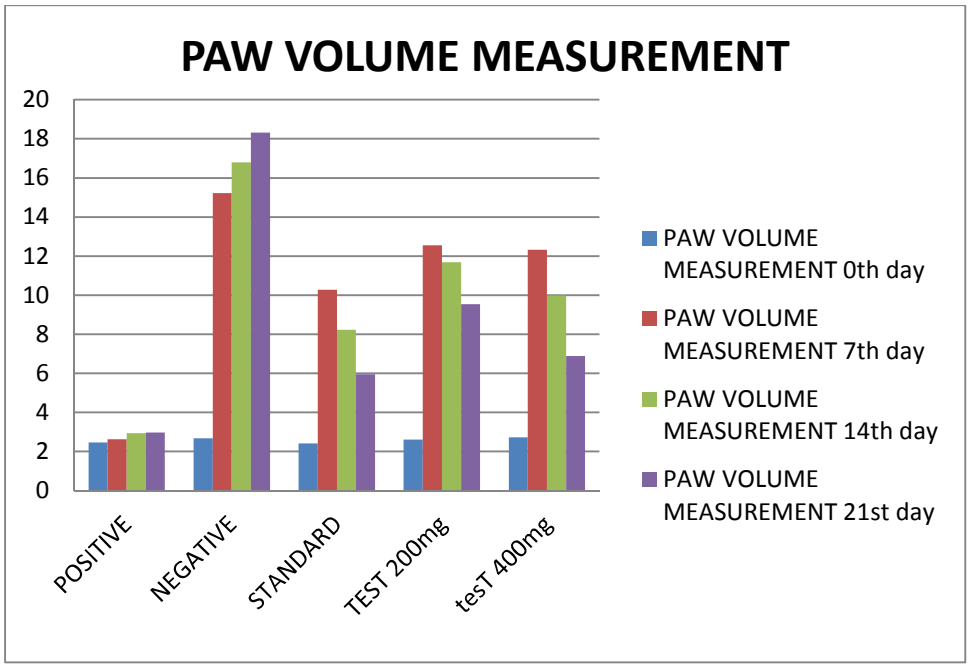
PAW VOLUME MEASUREMENT

TABLE NO: 20 MEASUREMENT OF PAW VOLUME USING PLETHYSMOGRAPH- ADJUVANT-INDUCED ARTHRITIC RATS

Treatment	0 day	7th day	14th day	21st day
Group I (Control)	2.46±0.54	2.63 ±0.45**	2.94 ±.14**	2.98±0.32**
Group II (Arthritic control)	2.68 ± 0.21	15.22 ± 0.15*	16.79 ± 0.32*	18.32± .142*
Group III Diclofenac sodium	2.41±0.34	10.27±0.17***	8.23±0.14***	5.96 ± 0.13***
Group IV (200 mg/ kg)	2.62±0.113	12.56±0.213***	11.69 ± 0.16***	9.54±0.019***
Group V (400mg/kg)	2.73±0.21	12.32± 0.14***	9.98 ± 0.145***	6.89±0.018***

*p< 0.05, **p < 0.01, ***p < 0.001 as compared to the positive and arthritis control. The data was analyzed using one way analysis of variance (ANOVA) followed by Tukey HSD Test.

FIG NO: 35 PAW VOLUME MEASUREMENT- ADJUVANT INDUCED ARTHRITIC RATS



*p< 0.05, **p < 0.01, ***p < 0.001 as compared to the positive and arthritis control. The data was analyzed using one way analysis of variance (ANOVA) followed by Tukey HSD Test.

TABLE NO: 21 PERCENTAGE INHIBITION OF PAW VALUE - ADJUVANT INDUCED ARTHRITICRATS

Treatment	7th day	14th day	21st day
Group I (Positive control)	**0**	**0**	**0**
Group II (negative control)	**0**	**0**	**0**
Group III (Diclofenac sodium)	32.52	50.98	67.46
Group IV (200 mg/ kg)	17.47	30.37	47.92
Group V (400mg/kg)	19.05	40.55	62.39

The Percentage inhibition of paw volume of all the group of was found. The standard group, test dose 200mg/kg and test dose 400mg/kg were studied for the different intervals of time. The standard group showed maximum percentage inhibition and the test dose 400mg/kg showed better percentage inhibition than test dose 200mg/kg. It indicates that 400mg/kg test dose showed significant arthritic effect on rats.

RADIOGRAPHY STUDY

The proximal inter phalangeal joints were removed and washed with saline and stored in 10% formalin. These joints were subjected to radio graphical studies.

The radiographic images of hind limb of rats were taken and discussed as follows.

- Group I : Normal control group showed no changes in the joints.
- Group II : Arthritic control group showed destruction of bones at joints, narrowing of joints and swelling of soft tissues are seen.
- Group III : Standard group (Diclofenac sodium) showed no destruction of bones at joint, swelling of soft tissues are reduced to normal.
- Group IV : Test dose I (200mg/kg) showed mild destruction of bones and swelling of soft tissues.
- Group V : Test dose II (400mg/kg) showed the mild changes in the joint and complete reduced swelling of soft tissues.

RADIOGRAPHY OF BONE

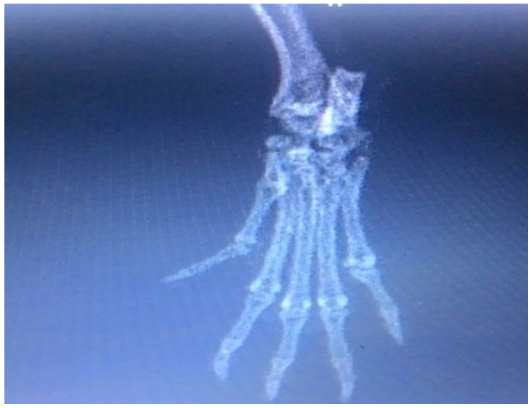


FIG NO: 36 POSITIVE CONTROL

FIG NO: 37 NEGATIVE CONTROL

FIG NO: 38 STANDARD (DICLOFENAC SODIUM)

FIG NO: 39 TEST DOSE 200mg/kg

FIG NO: 40 TEST DOSE 400mg/kg

HISTOPATHAOLOGICAL EXAMINATION OF BONE

- Group I : Normal control group showed normal cartilage.
- Group II : Arthritic control group showed erosion of bone and formation of edema.
- Group III : Standard group (Diclofenac sodium) showed normal cartilage and no formation of edema occurs.
- Group IV : Test dose I (200mg/kg) showed mild erosion of bones.
- Group V : Test dose II (400mg/kg) showed normal cartilage and absence of edema.

HISTOPATHOLOGY OF BONE

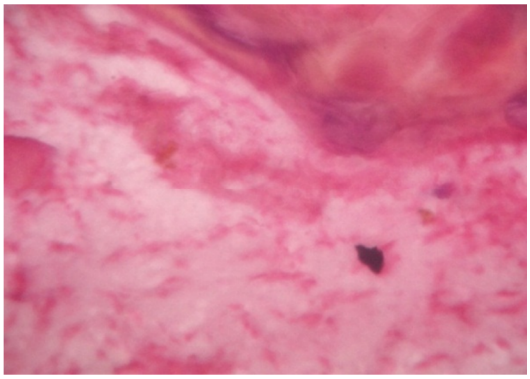


FIG NO: 41 POSITIVE CONTROL

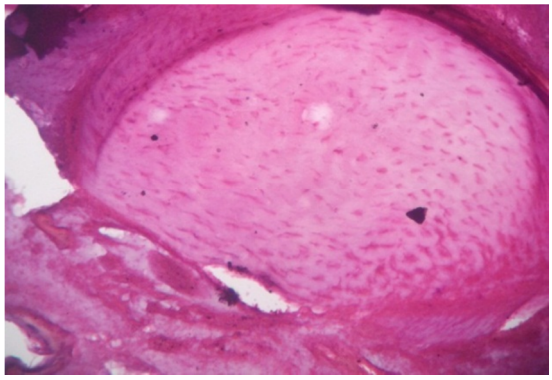


FIG NO: 42 ARTHITIC CONTROL

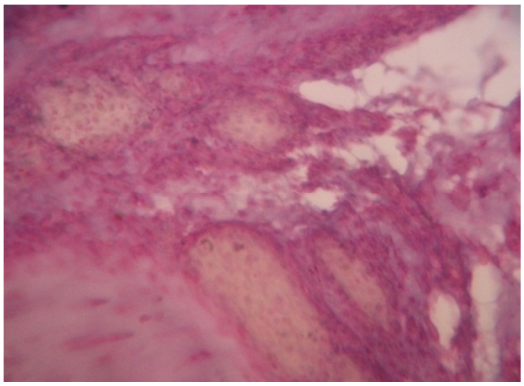


FIG NO: 43 STANDARD (DICLOFENAC SODIUM)

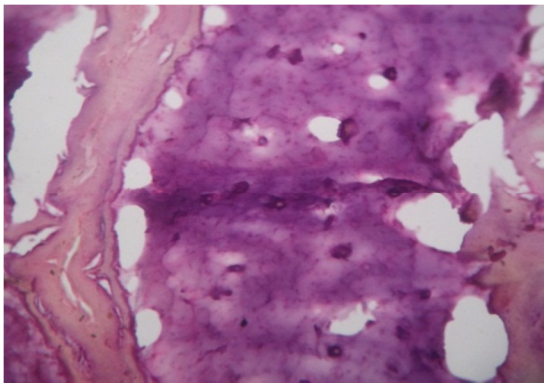


FIG NO: 44 TEST DOSE 200mg/kg

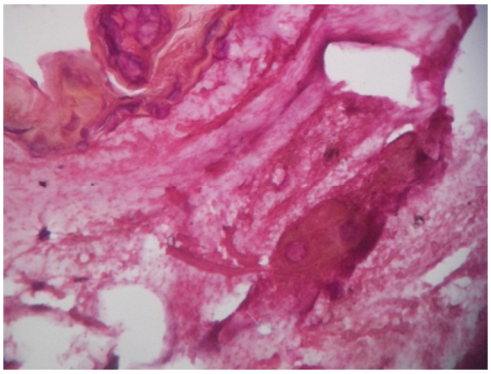


FIG NO: 45 TST DOSE 400mg/kg

Isolation and Identification of Lupeol

11. ISOLATION AND IDENTIFICATION OF LUPEOL

Since lupeol was found to possess anti-inflammatory and anti-arthritic activity[75] an attempt was made to isolate and identify the lupeol from the Ethanol extract of *Sterculia foetida* Linn., Lupeol is a pentacyclic triterpenoid derivative. Chemically it is hexamethyl-1-prop-1en-2yl-hexadecahydrocyclopenta-chrysen-9-ol. The molecular formula of lupeol is $C_{30}H_{50}O$ and the molecular weight is 426.73g.mol. It has several medicinal properties.

Occurrence

Lupeol is found in a variety of plants, including mango, *Acacia visco,* and *Abronia villosa*. It is also found in dandelion coffee.

Chemical properties

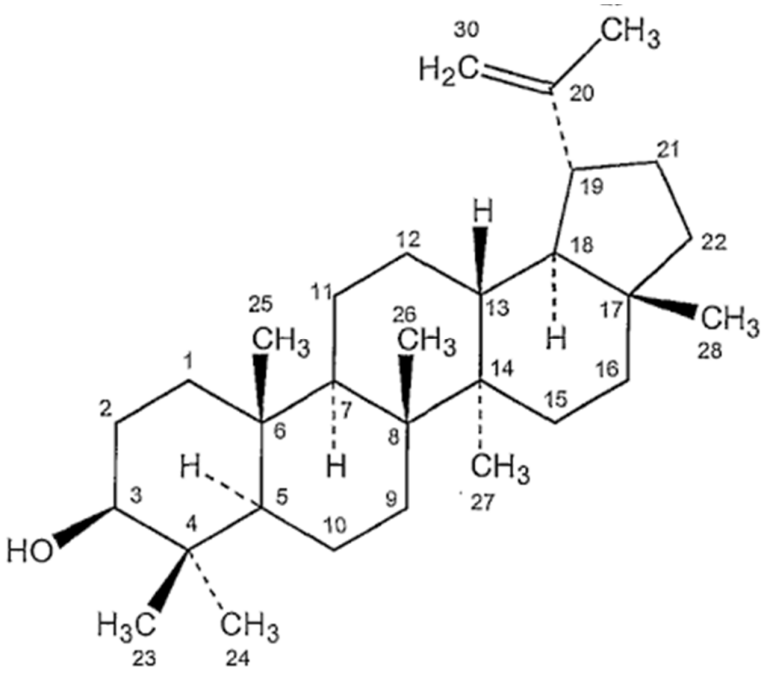


FIG N0: 46 STRUCTURE OF LUPEOL

11.1. Materials and method

Extract used : Ethanol extract

Method : Column chromatography

Procedure

The separation of compounds was achieved by column chromatograghy[73]. Then weighed quantity of ethanolic extract was subjected in to the column chromatography on a silica gel, the column is packed using 100% n-Hexane. The solvent system used for the elution is Ethanol : Ethyl acetate (50:50). Eluents were collected in 25ml aliquots, evaporated to dryness and was further subjected to purification. This compound appeared as white needles and was subjected to spectral analysis.

Spectral studies

The isolated compound was subjected to IR, NMR and GC-MS analysis.

Infra-red spectroscopy

It is the absorption spectrum of different IR frequencies by a sample positioned in the path of an IR beam. It is carried out for the determination of functional groups in the sample. The commonly used method is pressed pellet technique for solid sample and Nujol mull technique for liquid sample. With this technique the isolated sample is investigated for the functional groups.

NMR SPECTROSCOPY

It is technique in which position of the Hydrogen and Carbon atoms were identified. NMR spectra were acquired on a Varian Unity Pulse 600 MH_Z instrument using standard pulse sequences at ambient temperature. Chemical shifts are given in δ (ppm) and coupling constants are reported in H_Z. The samples were diluted with water : acetonitrile : methanol (1:2:2) and stock solution were prepared. The sample was introduced using syringe pump at a flow injection rate of 120µl/min. sample was studied for both H1 NMR and C13 NMR respectively.

GC-MS ANALYSIS

The extract was subjected to GC-MS analysis. Chromatographic separation was carried out for the isolated compound. Helium used as a carrier gas with a flow rate of 1 ml/min in the split mode. An aliquot amount of sample was injected into the column with the injector, heater at 250ºC. The data analysis of compound was noted in mass spectra in which the detector operated in scan mode from 20 to 600 atomic mass units (amu). Identifications were based on the molecular structure, molecular mass and calculated fragmentations.

11.2. RESULTS AND DISCUSSION

PHYSICAL AND CHEMICAL PROPERTIES OF ISOLATED COMPOUND

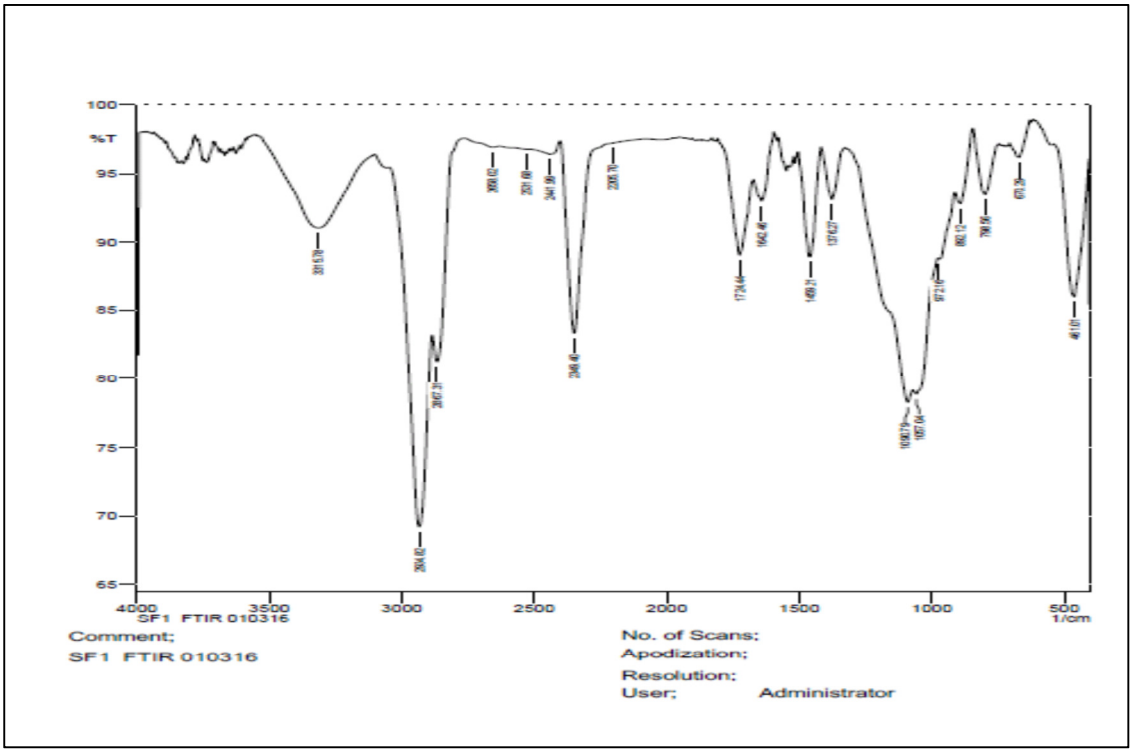
Colour	Greenish white
Nature	Solid (needle shaped crystals)
Melting point	215º C
Solubility	Soluble in water, chloroform, ethanol and methanol
Chemical test	**Libermann Burchards Test**
	Formation of brown indicates presence of triterpenoids.
TLC Solvent system	Ethyl acetate : Toluene : Methanol : Formic acid (6:2:1:1)
R_f value	0.68

IR SPECTROSCOPY

TABLE NO: 22 IR SPECTRUM DATA FOR ISOLATED COMPOUND

S.no.	Observation	Observed wave number (cm^{-1})	Standard wave[76] number (cm^{-1})
1.	**O-H Stretching**	3315.78	3434.61
2.	**C-H Stretching alkane**	2934.82	2947.33
3.	**C=C Stretching alkene**	1642.46	1640.99
4.	**C-H stretching alkyl**	1459.21	1463.14
4.	**C-H alkene**	2867.31	2873.33
5.	**CH_3 Stretching**	1376.27	1382.55
6.	**C-C Stretching**	1057.04	1062.72

FIG NO: 47 IR SPECTRUM FOR ISOLATED COMPOUND



The isolated compound was subjected to IR studies and the results were compared with the standard lupeol for reference.

NMR SPECTROSCOPY

Molecular formula $C_{30}H_{50}O$

M+ peak 424 [M+];

1H NMR

7.21 (m - 1H, H-6), 7.19 (s- 1H), 4.16 (s - 1H), 4.14 (s - 1H), 3.62 (tdd - OH, H-3), 1.27 (s – 3H), 1.19 (s- 3H), 1.07 (s – 3H), 0.99 (s – 3H).

13C NMR

212.8 (C- 3), 150.4 (C-20), 108.8 (C-29), 59.3 (C-5), 58.0 (C-9), 53.1 (C-18), 42.6 (C-19), 42.2 (C-17), 41.6 (C-4), 41.4 (C-14, 8), 40.4 (C-22), 39.7 (C-1), 36.0 (C-10, 16), 35.6 (C-13), 35.1 (C-2), 33.1(C-7), 32.3 (C-23), 32.0 (C-24), 30.2 (C-15), 29.7 (C-21), 29.4 (C-12), 22.5 (C-11), 21.0 (C-30), 20.2 (C-28), 18.9(C-25), 18.5 (C-6), 18.0 (C-26), 15.1 (C-27).

The mass spectral data of compound gave a molecular ion peak at m/z 424 corresponding to its (M)+ ion suggesting the molecular formula as $C_{30}H_{50}O$.

The 1H NMR spectra of compound showed the presence of seven methyl singlets at 7.12, 7.19, 4.16, 1.27, 1.19, 1.07 and 0.99.The 1H NMR spectra of compound also showed the presence of two protons appeared at 3.62 and 4.14 as singlets representing the exocyclic double bond protons. The 13C NMR spectrum of compound showed a saturated carbonyl group at 212.8 and the alkene carbons at 150.4 and 108.8 suggesting the presence of a lupane triterpene having a carbonyl group in its structure. The 1H and 13C NMR values for all the protons and carbons were assigned on the basis of COSY, HMQC and HMBC correlations as reported 15-17 and were given in materials and methods. Considering lupane skeleton for the compound together with the absence of a hydroxyl group and the appearance of a carbonyl group in the 13C NMR spectral data suggested the presence of a keto functional group and its presence was identified at C-3 position by the key HMBC correlations.

FIG NO: 48 H1 NMR SPECTRUM FOR ISOLATED COMPOUND

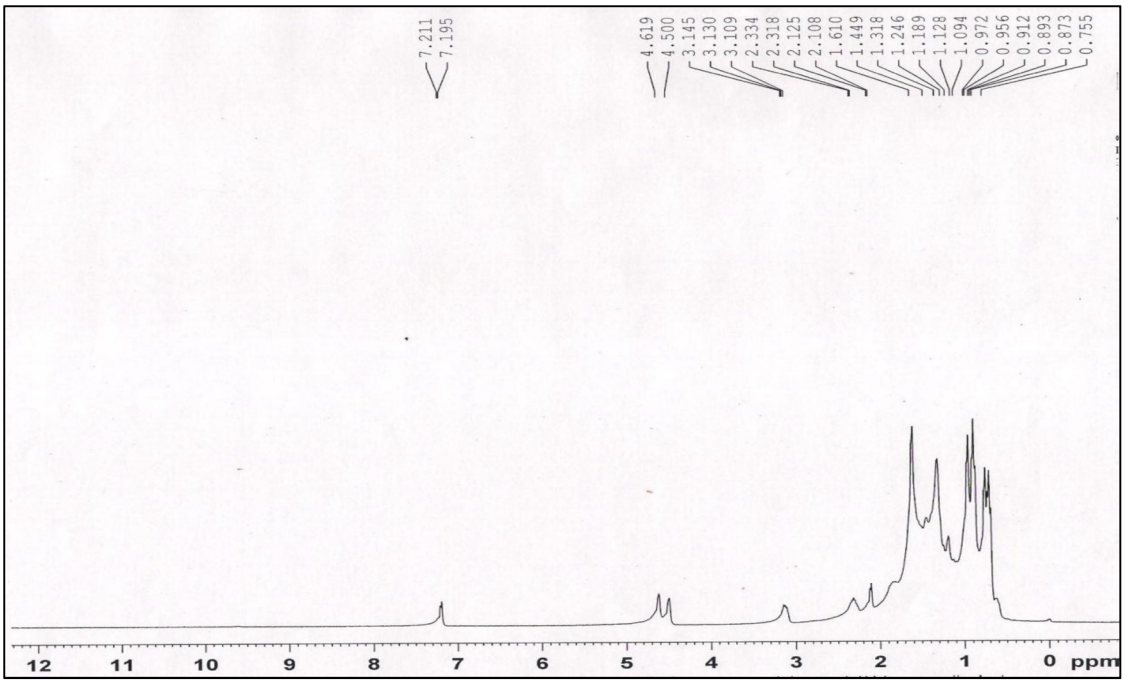
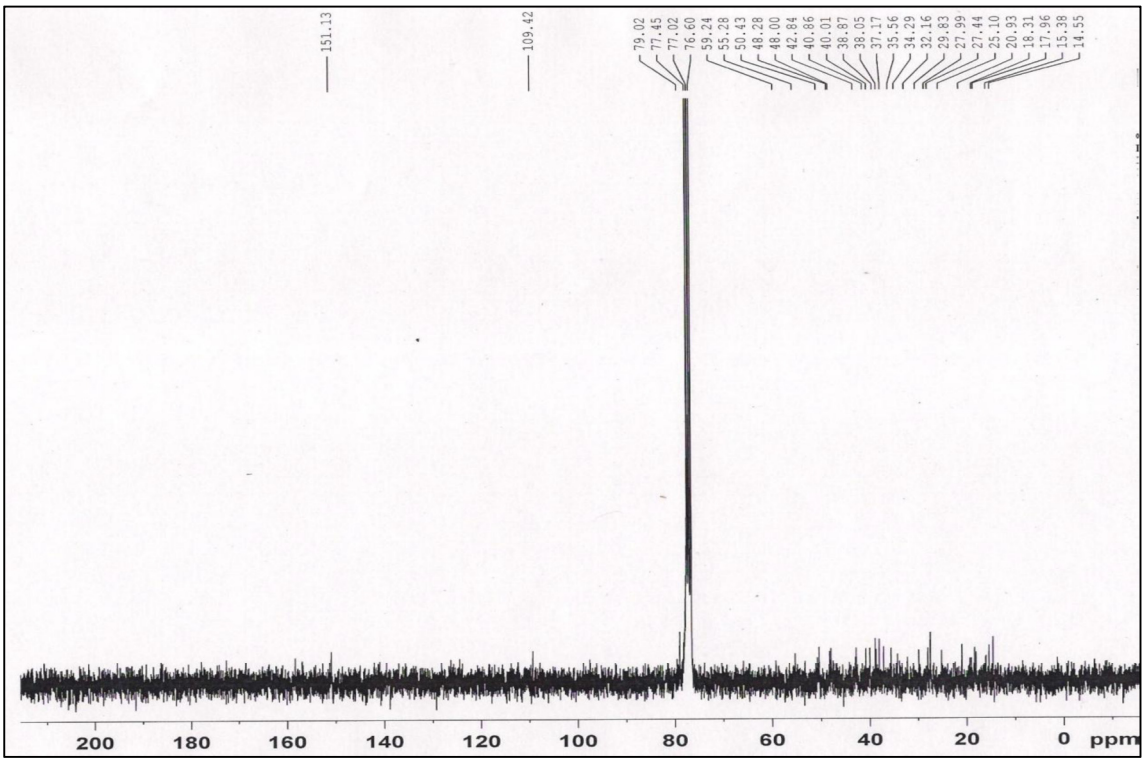


FIG NO: 49 C13 NMR SPECTRUM FOR ISOLATED COMPOUND

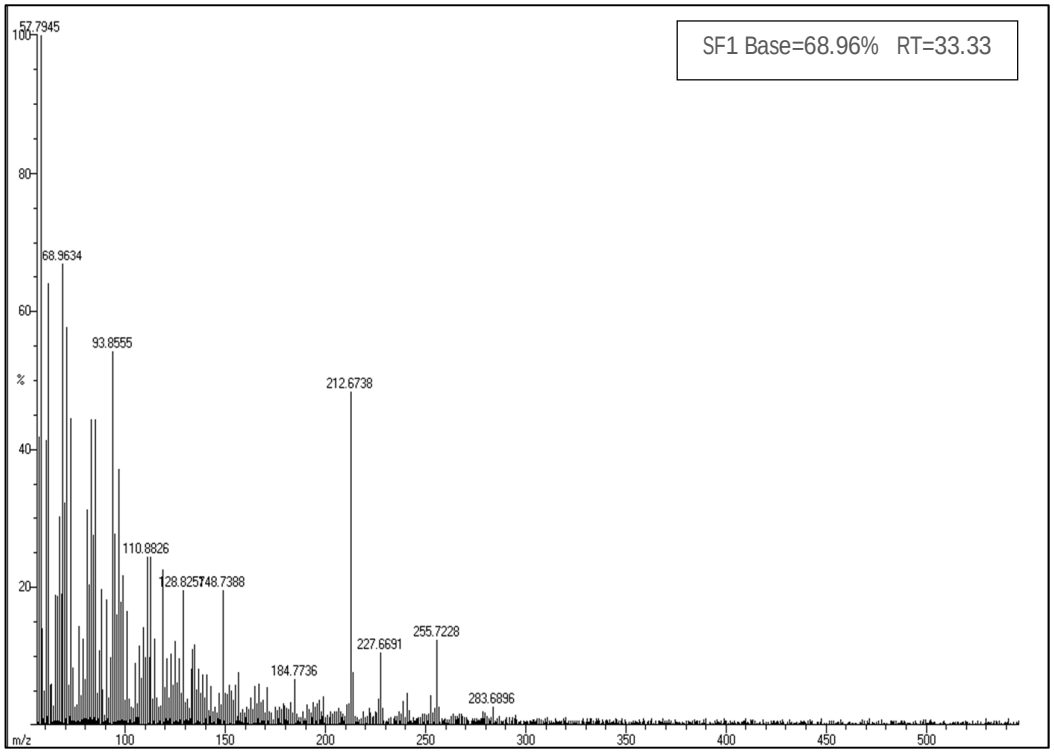


GC-MS ANALYSIS

The compound present in the ethanolic extract were identified by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the ethanol extract is listed as follows.

Retention time	33.33
Molecular formula	$C_{30}H_{50}O$
Molecular weight	426.73
Peak area %	68.14%

FIG NO: 50 GC-MASS SPECTRUM FOR ISOLATED



CONCLUSION FOR ISOLATION

Based on the physical, chemical properties and the spectral studies such as IR, NMR and GC-MS results the isolated compound was found to be lupeol. The isolated lupeol was compared with that of standard lupeol. This lupeol is considered to be responsible for the pharmacological actions.

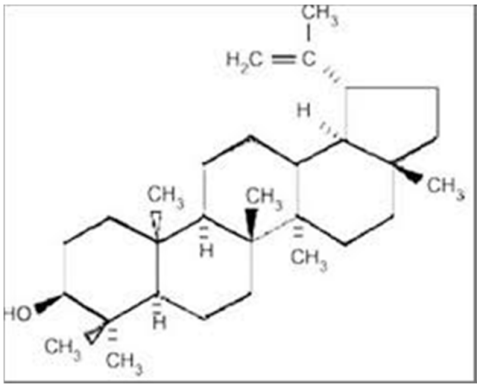


FIG N0: 51 STRUCTURE OF ISOLATED COMPOUND

Summary and Conclusion

12. SUMMARY AND CONCLUSION

Among the world wide population arthritis affects 15% of people. The prevalence of arthritis is increasing throughout the world. In recent years, ethnobotanical and traditional uses of phytoconstituents from plant origin received much attention as they are very effective and safe for human use. Man was completely depending on the natural medicine because of reduced side effects. Herbal medicine is a major component in all indigenous traditional medicine and a common element in Ayurveda, Siddha, Homeopathy and Naturopathy systems of medicine. Hence the present study is an attempt to evaluate the anti-arthritic activity on the bark of *Sterculia foetida* Linn.,

PHARMACOGNOSTICAL STUDIES

The pharmacognostical studies on the bark of *Sterculia foetida* Linn., was carried out, which showed the unique features of the bark which is used to differentiate it from other species.

Macroscopical studies states the characteristic features of bark like grey colour, smooth in outer and fibrous in inside, single quill and smooth texture.

The anatomical studies of the bark showed the presence of cork, cortex, sclerides, phloem and medullary rays.

Powder analysis of the bark showed the presence of parenchyma, fibers, sclerides, calcium oxalate crystals, etc,.

Physiochemical studies various physiochemical constants were evaluated such as ash values, extractive values, foreign organic matter, foaming index, swelling index and loss on drying.

These pharmacognostical parameters evaluated are useful for the establishment of standards of bark which is essential for its identity and purity.

Phytochemical studies

In phytochemical study, the powdered bark is successively extracted with n-hexane, chloroform, ethyl acetate and ethanol by using soxhlet apparatus.

Preliminary phytochemical investigation was done for the powdered bark and all the extracts. It was found to contains flavonoids, triterpenoids, alkaloids, phenolic compounds, steroids, etc,.

Quantitative estimation of the phytoconstituents is carried out for alkaloids, steroids, phenolic compounds and flavonoids.

Fluorescence analysis was done to find out characteristic fluorescent substance present in the powdered bark and all the extracts, and no fluorescent substance was found.

High performance thin layer chromatography (HPTLC) was performed with ethanolic extract of bark of *Sterculia foetida* Linn., and the finger print showed the presence of 12 peaks helpful in the quantitative and qualitative identification of phytoconstituents of *Sterculia foetida* Linn., bark.

***In vitro* studies**

All the extracts were subjected to *in-vitro* antioxidant activity and *in-vitro* anti-arthritic activity. The ethanolic extract showed the maximum antioxidant potential and maximum inhibition in the protein denaturation method. Hence the ethanolic extract was chosen for the *in-vivo* studies.

Pharmacological activity

Acute toxicity studies were carried out in the Wistar albino rats as per the OECD guidelines. The Ethanol extract was found to be safe up to the dose of 2000mg/kg. Therefore $1/10^{th}$ of the dose (200mg/kg) and $1/20^{th}$ of the dose (400mg/kg) were selected.

In-vivo anti-arthritic activity was carried out for ethanolic extract in Wistar albino rats by using complete freund's adjuvant induced arthritis model. Ethanolic

extract of dose 200 and 400mg/kg. were given orally to the rats. It is compared with standard Diclofenac sodium. Various parameters were studied for the evaluation of anti-arthritic activity. The ethanolic extract at the dose 400mg/kg. showed significant anti-arthritic effect on the arthritis induced rats which was comparable with that of the standard.

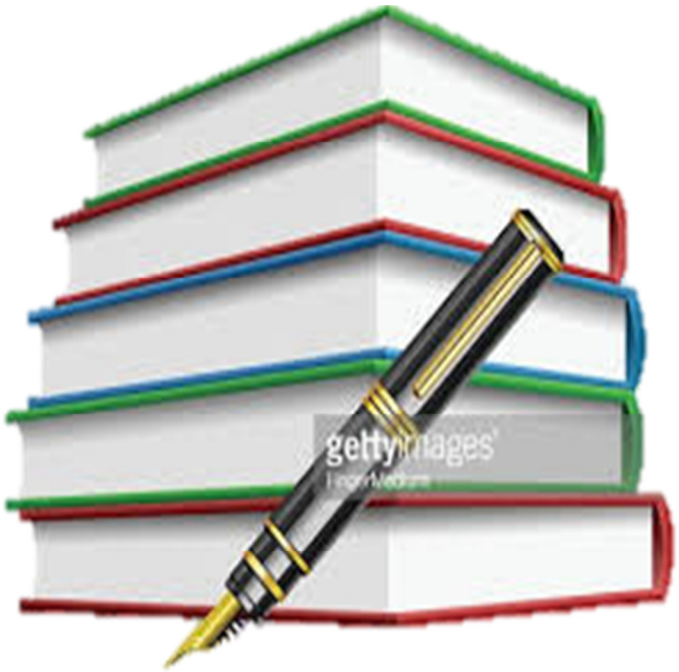
Isolation and Identification of Lupeol

The Lupeol was isolated by column chromatography method and identified by spectral analysis such as IR, NMR and GC-MS methods. The lupeol is pentacyclic tri terpenoid derivative which is having anti-inflammatory and anti-arthritic effect. Hence the isolated compound lupeol was considered to be responsible of pharmacological actions.

CONCLUSION

From the above studies it can be concluded that the Pharmacognostical standards generated will be useful for the proper identification of the plant that could be made use of, those who deal with the species and also in the quality assurance of the plant species with the support of Phytochemical and *in-vitro* Pharmacological studies, ethanolic extract was selected and subjected to *in-vivo* anti-arthritic activity. The ethanolic extract at the dose of 400mg/kg showed significant anti-arthritic activity which was comparable with that of the standard. Lupeol was isolated from the ethanolic extract and identified by spectral studies.

Further studies are focused on *in-vivo* pharmacological activity of isolated compound and its structural activity relationship.



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Annexures

ANNEXURES

LIST OF PAPERS PUBLISHED

S.no.	Name of the article	Publication/journal name	Status of the article
1.	Review of *Sterculia foetida* Linn.,	RJPP	Published-2015
2.	Phytochemical Screening on the successive extracts of *Sterculia foetida* Linn.,	IJIR	Published-2016
3.	Establishment of Pharmacognostical standards for the bark of *Sterculia foetida* Linn.,	RJPP	Accepted-2016

LIST OF POSTER PRESENTED IN CONFERENCE

S.no.	Title	Conference name	Year
1.	*In-vitro* antioxidant and *in-vitro* anti-arthritic activity on the bark of *Sterculia foetida* Linn.,	67^{th} International Pharmaceutical Congress	2015

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Fax: 044-22381314
Date: 14-07-2015

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Certified that the following plant drug taken for Dissertation project work by **Ms.M.Kavitha.,** M.Pharm. final year, Department of Pharmacognosy, Madras Medical College, Chennai is identified and authenticated through Visual inspection, Experience, Education & Training, Organoleptic characters, Morphology and Taxonomy methods as

Sterculia foetida Linn. (Sterculiaceae), Stem Bark

MEDICINAL BOTANY ★ CHENNAI 600 047 ★ National Institute of Siddha

Authentication Certificate No: NISMB1732015

Reference:

D.J.Mabberley, The Plant-Book, A portable dictionary of the vascular plants, Second edition, P.683

Authorized signatory: 14/7/15

Dr. D. ARAVIND, M.D.(s),M.Sc.,
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This is to certify that Ms. **M. KAVITHA**, M.Pharm II year, Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai – 600003 had submitted her protocol (Part B Application) 17/243/CPCSEA for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai – 600003.

TITLE: PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTI-ARTHRITIC ACTIVITY ON THE BARK OF *Sterculia foetida* Linn.,

The Animal Ethical Clearance Committee experts screened her proposal No: 17/243/CPCSEA and have given clearance in the meeting held on 10/08/2015 at Dean's Chamber in Madras Medical College, Chennai – 600003. Her study involves only Wistar albino rats.

Signature

9/3/2016

Dr. S.K. SEENIVELAN, B.V.Sc.,
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The paper has been published after getting reviewed by reviewers.

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